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# PNEUMOCOCCUS CULTURES IN WHOLE FRESH BLOOD.

## I. The Retardative Effect of the Blood of Immune Animals and the Mechanism of the Phenomenon.

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(Received for publication, November 29, 1919.)

The failure to realize more fully in the field of specific immunotherapy the expectations that were engendered by the discovery of specific antitoxins for diphtheria and tetanus is due, no doubt, in large measure to the lack of definite and complete knowledge of the mechanisms of immunity reactions.

It is noteworthy that, with few exceptions, a multiplicity of theories has been evolved with respect to the identity and nature of the factors and processes operative in natural recovery from each infectious disease to which man is subject. This is true of infections by the pneumococcus, notwithstanding the fact that these infections lend themselves, to an exceptional degree, to experimental study. It would be difficult to mention a more striking example of antibacterial immunity than that conferred upon a susceptible animal by the administration of antipneumococcus serum. Moreover, some of the species of laboratory animals are highly susceptible to pneumococcus infections, while others are refractory to a corresponding degree, thus making it possible to study any type of immunity—natural immunity or active or passive acquired immunity.

The protective power of antipneumococcus serum has been ascribed to different factors by various investigators. Thus Mennes,[1] Boehncke and Mouriz-Riesgo,[2] and others advanced the theory that antipneumococcus serum possesses marked

[1] Mennes, F., *Z. Hyg. u. Infectionskrankh.*, 1897, xxv, 413.

[2] Boehncke, K. E., and Mouriz-Riesgo, J., *Z. Hyg. u. Infectionskrankh.*, 1915, lxxix, 355.

antitoxic properties, while Neufeld and Rimpau[3,4] claim that pneumococcus immunity depends entirely upon the bacteriotropic substances of the immune serum and the phagocytic cells of the host. More recently it has been pointed out by Wright[5] that the whole uncoagulated blood of man is, in certain instances, highly pneumococcidal, and a case is cited in which 1 cc. of blood killed from 600,000 to 1,000,000 organisms. Heist and his collaborators[6,7] have extended Wright's observations, including experimental animals. It is claimed by them that the pneumococcidal capacity of the whole uncoagulated blood is a direct index of resistance to pneumococcus infection and that the virulence of the organisms is directly proportional to their insusceptibility to this killing property of the blood. Cole and his coworkers[8] and Winternitz and Kline[9] believe that any or all of the factors mentioned above cannot be held entirely responsible for immunity to pneumococcus infection and that an unidentified factor is essential.

The work reported in the present paper concerns the alleged pneumococcidal property of the whole blood of immune animals and it is therefore desirable to state briefly a few points concerning antipneumococcus serum which are of interest in this connection. Pneumococci can be grown for an unlimited number of generations in the most potent antipneumococcus serum. This treatment profoundly affects the organisms, as shown by Stryker,[10] yet there is no evidence that any of the organisms are killed by the serum. The same is true of the blood serum of naturally immune animals. Pigeons, for example, possess practically an absolute immunity to pneumococcus infections, while the fresh serum of pigeons is a good culture medium for pneumococci. The defibrinated blood of immune animals is only a slightly less favorable medium than the serum. On the other hand, pneumococci do not multiply in the blood stream of refractory animals and the intravenous administration of antipneumococcus serum sterilizes,

[3] Neufeld, F., and Rimpau, W., *Deutsch. med. Woch.*, 1904, xxx, 1458.

[4] Neufeld, F., and Rimpau, W., *Z. Hyg. u. Infectionskrankh.*, 1905, li, 283.

[5] Wright, A. E., On pharmo-therapy and preventive inoculation applied to pneumonia in the African native, New York, 1915, 78.

[6] Heist, G. D., Solis-Cohen, S., and Solis-Cohen, M., *J. Immunol.*, 1918, iii, 261.

[7] Heist, G. D., and Solis-Cohen, S., *J. Immunol.*, 1919, iv, 147.

[8] Avery, O. T., Chickering, H. T., Cole, R., and Dochez, A. R., Acute lobar pneumonia. Prevention and serum treatment, Monograph of The Rockefeller Institute for Medical Research, No. 7, New York, 1917.

[9] Winternitz, M. C., and Kline, B. S., *J. Exp. Med.*, 1915, xxi, 320.

[10] Stryker, L. M., *J. Exp. Med.*, 1916, xxiv, 49.

within a few minutes, the blood of an animal having a pneumococcemia. This last phenomenon has been ascribed to the agglutinating and bacteriotropic properties of the immune serum and the phagocytic cells of the host rather than to a bactericidal action of the serum.[11] Since the reactions here concerned take place within the whole blood, it would, in the light of the reports of Wright and Heist and his coworkers, be reasonable to suppose that the bactericidal property of the whole circulating blood of the immune animal plays an important part in this phenomenon.

## EXPERIMENTAL.

*Technique of the Whole Blood Test.*[5, 6]—Sets of five or more capillary glass tubes about 1 mm. in diameter are filled respectively, by capillary attraction, to a fixed point with graded dilutions of an 18 hour blood broth culture of pneumococci, the culture being immediately drawn from the tubes by touching the filled ends with sterile gauze. This leaves a coating of culture on the walls of the tubes, the number of bacteria left behind varying with the dilution of the culture. When the tubes have dried in the air for a few minutes they are filled to the fixed point with the blood to be tested by touching them to a drop of blood as it flows from a punctured vein. The tubes are sealed by dipping them into melted paraffin (both ends may be sealed, the loaded end first). They are incubated at 37°C. for any desired length of time and then examined by making films of the contents, staining, and examining with the microscope, or by making cultures in suitable medium. It is expedient to make five or more dilutions of the culture, varying from undiluted culture to a dilution of 1 : 1,000, broth being used as the diluting fluid. Three or more sets of capillary tubes should be prepared for each test in order that a set of dilutions may be examined after different incubation periods, varying from 1 to 96 hours as the test may demand.

For microscopic examination the entire contents of a tube are blown upon a slide and spread thinly over the surface. The films are allowed to dry in the air, fixed in methyl alcohol, and stained with Manson's stain. To avoid overlooking the bacteria, especially when they are not evenly distributed, it is necessary to go over the whole preparation with great care. When diplococci are found in the preparations it indicates that multiplication has occurred, for on control slides made immediately after the tubes are loaded, bacteria are never found, except when undiluted culture has been used to charge the

[11] Bull, C. G., *J. Exp. Med.*, 1915, xxii, 457.

tubes, and even here the slides are usually negative. Cultures are made from the tubes by blowing the contents into the condensation fluid of freshly prepared rabbit blood agar slants, and the clot, when whole blood is being tested, is broken up and spread over the surface of the slant. The inoculated tubes are examined after from 24 to 48 hours incubation.

In determining the relative effects of the blood of different animals on the growth of the culture it is essential that the tests should be prepared simultaneously and with the same dilutions of culture. All other conditions of the tests must be the same in each instance, for slight variations will give incorrect results. It is well to run a control set of tubes with normal rabbit blood in order to establish the viability and vigor of the culture.

Any digression from these general directions for technique will be pointed out in the protocols.

The distinctive and important feature of the tests is that the organisms are brought into contact with the undiluted blood or other fluid to be tested.

*Experiment 1.*—In these tests with pigeon blood the capillaries were charged with dilutions of culture ranging from undiluted culture to a dilution of 1 : 625. Sets of tubes were examined after from 1 to 72 hours incubation. Control tests with normal rabbit blood were made in each instance. Fresh pigeon serum was also used as a control.

Pigeon blood, as compared with rabbit blood, greatly retards the multiplication of pneumococci. Capillaries charged with undiluted culture and filled with rabbit blood are positive on microscopic examination after from ½ to 1 hour incubation, while those filled with pigeon blood do not become positive until after an incubation period of from 6 to 18 hours. Capillaries with a 1 : 625 dilution of culture become positive in rabbit blood after from 5 to 7 hours incubation, while in the tubes filled with pigeon blood organisms cannot be found until after about 72 hours. Tubes with intermediate dilutions of culture become positive after a corresponding incubation period. All the tubes give positive cultures at any time during the test (Table I).

A negative microscopic examination does not necessarily mean that the pneumococci have been killed, but a negative culture is essential to such a conclusion. As far as the rabbit and pigeon are concerned, the rapidity with which the cultures develop may be taken as a fair index of their relative susceptibility to infection by these organisms.

TABLE I.

*Pneumococcus Cultures in Fresh Pigeon Blood; Microscopic versus Culture Test.*

| Dilution of culture with which capillary was charged. | Incubation. | Microscopic examination. | Culture on rabbit blood agar. |
|---|---|---|---|
| | *hrs.* | | |
| Undiluted. | 1 | − | + |
| 1 : 5 | 1 | − | + |
| 1 : 25 | 1 | − | + |
| 1 : 125 | 1 | − | + |
| 1 : 625 | 1 | − | + |
| Undiluted. | 6 | − | + |
| 1 : 5 | 6 | − | + |
| 1 : 25 | 6 | − | + |
| 1 : 125 | 6 | − | + |
| 1 : 625 | 6 | − | + |
| Undiluted. | 12 | + | + |
| 1 : 5 | 12 | − | + |
| 1 : 25 | 12 | − | + |
| 1 : 125 | 12 | − | + |
| 1 : 625 | 12 | − | + |
| Undiluted. | 18 | + | + |
| 1 : 5 | 18 | + | + |
| 1 : 25 | 18 | − | + |
| 1 : 125 | 18 | − | + |
| 1 : 625 | 18 | − | + |
| Undiluted. | 42 | + | + |
| 1 : 5 | 42 | + | + |
| 1 : 25 | 42 | + | + |
| 1 : 125 | 42 | + | + |
| 1 : 625 | 42 | − | + |
| Undiluted. | 65 | + | + |
| 1 : 5 | 65 | + | + |
| 1 : 25 | 65 | + | + |
| 1 : 125 | 65 | + | + |
| 1 : 625 | 65 | + | + |

*Experiment 2.*—Comparative tests were made with the blood of a number of other laboratory animals. The microscopic method was used to determine whether growth had occurred, a positive result meaning that diplococci were found in the stained preparations. Sufficient cultures were made to eliminate the possibility of contaminations. Since the tubes always became positive in the order of the dilutions of culture with which they had been charged, only the results of the two extreme dilutions are given. Table II gives a summary of these tests.

Table II includes only normal individuals of the different species which are arranged in the order of the incubation (latent) period of

TABLE II.

*Latent Period of Pneumococcus Cultures in the Whole Blood of Different Animals.*

| Animal. | Length of time after which cultures are positive on microscopic examination. | |
|---|---|---|
| | Capillaries charged with undiluted culture. | Capillaries charged with 1:625 dilution of culture. |
| | *hrs.* | *hrs.* |
| Rabbit | ½–1 | 3–5 |
| Mouse | ½–1 | 4–6 |
| Cat | 1–2 | 5–6 |
| Guinea pig | 1–2 | 6–7 |
| Sheep | 2–3 | 6–7 |
| Man | 2–3 | 6–7 |
| Dog | 3–4 | 8–12 |
| Hen | 4–5 | 12–15 |
| Pigeon | 6–12 | 48–72 |

pneumococci in the respective bloods. It is seen that the rabbit, mouse, and cat come at the top of the list, the pigeon, hen, and dog at the other end, while the guinea pig, sheep, and man occupy intermediate positions. There is no doubt that in a general way this is the order of susceptibility to infection of these species to the pneumococcus, the rabbit and mouse being the most susceptible and the pigeon and hen the most resistant; but the relative positions of those falling in the middle zone are questionable. The cat, for example, should be farther separated from the rabbit and mouse, particularly on intravenous inoculation. Further tests showed that the method is not sensitive enough to classify normal individuals of the same species

with respect to susceptibility to pneumococcus infection. Experiments on vaccinated rabbits showed, however, that in this instance, an immunity reaction which is not made evident by other methods used to demonstrate antibodies[6, 7] can be detected by this method. The data recorded in Experiment 3 illustrate this point.

*Experiment 3.*—Each of two rabbits was given intravenously the killed cultures from two blood agar slants of pneumococci. On the 3rd day after the injection the rabbits were bled and the sera tested in the ordinary way for agglutinins and opsonins. Whole blood tests were set up at the time the blood was collected for the serum. The tests were repeated on each succeeding day, corresponding tests being made on normal rabbits as controls. Even on the 3rd day the blood of one of the treated rabbits caused a slight delay in the growth of the culture. On the 4th day the blood of both rabbits caused a definite lengthening of the latent period and this effect rapidly increased on the succeeding days, while agglutinins and opsonins were not demonstrable in the serum, by ordinary methods, until the 6th day, although 50 per cent serum was present in the tests.

As may be inferred from Experiment 3, active immunization of rabbits against pneumococci confers upon their blood the property of retarding the growth of these organisms, the actively immune rabbit conforming to the normal pigeon in this respect. Further experiments showed that passive immunity gives identical results. Guinea pigs and rabbits were used in these experiments.

The foregoing experiments show that, in a general way, the rapidity with which pneumococci multiply in the fresh whole blood of various animals is directly proportional to the susceptibility of these animals to infection by the pneumococcus. Slight differences in susceptibility cannot, at least in every instance, be detected by this method. The cat, for example, is many times more resistant than the rabbit to infection with pneumococci on intravenous inoculation, while the whole blood test would indicate that the cat is only slightly more resistant. It is also shown that natural and artificial immunity —both active and passive—are similar with respect to this test. Finally, the conclusion that the fresh uncoagulated blood of immune animals is highly pneumococcidal[5, 6, 7] is doubtful, since the mixtures did not become sterile during the course of the experiments and multiplication of the organisms always occurred when incubation was sufficiently prolonged. On the other hand, it cannot be concluded

that no killing occurs; but to prove positively that the blood possesses a pneumococcidal property it would be essential that some of the pipettes, for example those charged with the higher dilutions of culture, should become free of viable organisms. The phenomenon described is, however, closely associated with, and at least roughly parallel to, resistance to infection. It was thought, therefore, that the working out of the mechanism of the reaction would possibly give important information as to the nature of the factors and processes involved in pneumococcus immunity. A large number of experiments have been carried out with this end in view.

### *Microscopic Study.*

A microscopic study was made of cultures of pneumococci at different stages of development in the whole blood of the animals used in the foregoing experiments. Capillary tubes were charged with the culture and loaded with the fresh uncoagulated blood according to the method already described. Sufficient tests were prepared in each instance to permit of frequent examinations. Cultures made in the blood of normal rabbits were used as controls, rabbit blood being an excellent medium for pneumococci. The following points were noted: (1) the kind of clot formed by the different bloods, (2) distribution of diplococci in the medium, (3) chain and clump formation, and (4) phagocytosis.

*Clot Formation.*—The bloods of different species behave differently with respect to retraction of the clot within the capillary tubes. The rabbit clot retracts both transversely and longitudinally, usually pulling away from the walls of the tube within a short time. This results in a small clot, and a relatively large amount of free serum. The pigeon clot retracts very little in any direction and usually continues to fill the whole tube, and there is only a small amount of free serum. The hen clot usually retracts longitudinally, but less than that of rabbit blood, and pulls away from the walls of the tube to a much less degree than the latter. The other bloods come between these extremes with respect to retraction of the clot. This point will be referred to later, since it apparently plays at least a secondary part in the phenomenon under investigation.

*Distribution of the Organisms in the Culture.*—Three films were made from each capillary culture, (1) from the free serum, (2) from the serum adhering to the clot, and (3) from the clot. The serum was removed from the small culture tubes by means of a very fine capillary. The clot was blown upon a slide and moved over the surface in order to make a film of the serum adhering to the clot. The clot was then dried on filter paper and a preparation made of the dried clot. These preparations were made at all stages of the development of the cultures and it was found that in the earlier stages the pneumococci were multiplying actively in the serum, fewer organisms were in the serum adhering to the clot, and the clot itself was not invaded until the cultures were well developed. This was particularly true of the capillaries containing immune blood—pigeon, hen, or immune rabbit blood. The clot of normal rabbit blood was invaded earlier in the development of the culture. This distribution might have been predicted because (1) the seeding organisms are at the periphery of the clot, and (2), as has been stated, pneumococci grow readily in the most potent immune serum.

*Chain and Clump Formation.*—Just as in immune serum, pneumococci grow in chains and clumps in immune blood, the growth in this instance being largely in the serum after coagulation has taken place. In pigeon blood there is only a slight clumping but long chains are formed, while in hen blood large clumps develop and also long chains. Chains and clumps are formed in immune rabbit blood, the size of the clumps and the length of the chains varying with the degree of immunity and the incubation time. The first indication of the development in rabbits of the immunity response to an inoculation of the organisms is chain formation, and the second, clump formation. In highly immune blood, the growth is restricted to chains and clumps until the culture approaches maturity when the growth may become diffuse (uniformly distributed diplococci) within a short time. The chain and clump phases of the cultures remind one of local infections and the diffuse phase of general infections. Apparently growth does not become diffuse until the antibodies of the blood have been exhausted, or until the organisms have become less susceptible to the antagonistic influence of the blood.[12] The chain and clump phenom-

[12] Bull, C. G., *J. Exp. Med.*, 1916, xxiv, 7.

ena occur in pigeon blood, hen blood, in the blood of an immune rabbit or any other immunized animal, and to a less degree in normal dog blood. They do not occur in normal rabbit, mouse, or cat blood. This fact must be kept in mind when making microscopic examination of cultures in immune blood; otherwise, even massive clumps may be overlooked and the specimen pronounced negative.

*Phagocytosis.*—Phagocytosis of the pneumococci was first observed in preparations made from cultures in hen blood. For this purpose the cultures should be examined after from 4 to 5 hours incubation. Preparations from the serum adhering to the clot contain the greatest number of phagocyting cells (polymorphonuclear leucocytes). Phagocytosis occurs in any immune blood. In order to demonstrate this appearance to advantage in highly immune bloods (pigeon, immune rabbit) it was necessary to charge the tubes with concentrated suspensions of the organisms. This was effected by centrifugalization and resuspension of broth cultures. A tenfold concentration gave good results. When more dilute cultures are used, the number of phagocyting cells is small and satisfactory observations cannot be made.

The tentative hypothesis which we based on the above observations was that the development of the culture to the point of being positive on microscopic examination was retarded by chain and clump formation and possibly by phagocytosis. When the organisms are held tightly in a few clumps, instead of being uniformly distributed through the medium, there is less probability of observation with the microscope. Thus, of two preparations containing the same number of organisms, one clumps and the other evenly distributed diplococci, the first, barring accident, could easily be found negative and the second positive. Moreover, multiplication is probably actually retarded because of reduced nutrition or an accumulation of inhibitory substances in the interior of the clump. In regard to phagocytosis the notion was that either a certain number of the pneumococci were killed by the phagocytes or multiplication within the phagocytes was held in check for a time, thus retarding the development of the culture. Further work was planned to determine the part played by the leucocytes in the reaction.

*Phagocytosis and Retardation of Growth.*

If phagocytosis is essential in the retardation of the cultures in immune blood, it is evident that the phenomenon depends on two agents; *viz.*, opsonins and leucocytes. The reaction should not occur in the absence of either factor, and one of the factors being constant the retardation should be directly proportional, within certain limits, to the quantity of the other factor present. This conception of the problem guided the experiments recorded below.

*Defibrinated Blood.*—It is stated by Heist and his associates[6] that defibrinated immune blood (chicken) is as good a medium for pneumococci as defibrinated normal rabbit blood. We studied this point since it appeared that phagocytosis does not play an essential part in the retardation of the growth of the culture, since the leucocytes are present in defibrinated blood, presumably, as in whole blood. Immune rabbit blood was used in this study.

*Experiment 4.*—Capillary tubes were prepared as usual with (1) whole blood, (2) defibrinated blood, and (3) serum. The tests were set up simultaneously, the capillaries having been charged with the same dilutions of culture. The defibrinated blood and serum tests were prepared a few minutes after the blood had been collected by cardiac puncture. The defibrination was effected by shaking the blood with glass beads and the serum was collected by centrifugalizing a portion of the blood immediately after coagulation. The whole blood was collected from the ear vein.

By examining the cultures at short intervals it was found that the pneumococci multiply more rapidly in the defibrinated blood than in the whole blood, but growth in the serum is still more rapid than in the defibrinated blood (Table III).

*Leucocytes of Defibrinated Blood.*—Whole and defibrinated blood from the same animal were studied in regard to the number, type, and condition of the leucocytes present, total and differential counts being made. It was found that defibrination removes from one-half to three-fourths of the total leucocytes, and a large percentage of those remaining in the defibrinated blood are lymphocytes. In one instance the following observations were made: whole blood, total count 12,515, polynuclear cells 7,460, lymphocytes 4,880, mononuclear leucocytes 175; defibrinated blood, total count 3,900, polynuclear cells 835, lymphocytes 2,905, mononuclear leucocytes 160. In other instances the total count was not reduced to such a low figure but the

number of polynuclear cells remaining in the defibrinated blood was always relatively very low. It was further noted that the process of defibrination visibly injured a large percentage of the polynuclear cells.

In these experiments there was, at least roughly, a mathematical relation between the number of polynuclear cells present and the rate

TABLE III.

*Pneumococcus Cultures in the Whole Blood, Defibrinated Blood, and Serum of an Immunized Rabbit and in Normal Blood.*

| Dilution of culture with which capillary was charged. | Incubation. | Microscopic examination. | | | |
|---|---|---|---|---|---|
| | | Whole blood. | Defibrinated blood. | Serum. | Normal rabbit blood. |
| | *hrs.* | | | | |
| Undiluted. | 4 | − | + | + | + |
| 1 : 5 | 4 | − | − | + | + |
| 1 : 25 | 4 | − | − | − | + |
| 1 : 125 | 4 | − | − | − | + |
| 1 : 625 | 4 | − | − | − | − |
| Undiluted. | 8 | + | + | + | + |
| 1 : 5 | 8 | − | + | + | + |
| 1 : 25 | 8 | − | + | + | + |
| 1 : 125 | 8 | − | − | + | + |
| 1 : 625 | 8 | − | − | − | + |
| Undiluted. | 12 | + | + | + | |
| 1 : 5 | 12 | + | + | + | |
| 1 : 25 | 12 | + | + | + | |
| 1 : 125 | 12 | − | + | + | |
| 1 : 625 | 12 | − | − | + | |

of multiplication of the pneumococci. The points determined also eliminate the red cells as a decisive factor in the reaction.

*Relation between the Number of Cells Present and Retardation of Growth.*—The experiments given here corroborate the conclusion drawn from the study of the defibrinated blood; *i.e.*, the latent period of the culture is directly proportional to the number of leucocytes present.

*Experiment 5.*—Blood was collected from immune animals (immunized rabbits and hens) in oiled glassware, quickly centrifugalized at high speed, and the three layers (plasma, leucocytic cream, and cells) were separated, remixed in different proportions, and the mixtures run into culture-charged capillary tubes before co-

TABLE IV.

*Pneumococcus Cultures in Hen Blood, Serum, Plasma, and Equal Parts of Plasma and Leucocytic Cream.*

| Dilution of culture with which capillary was charged. | Incubation. | Microscopic examination. | | | |
|---|---|---|---|---|---|
| | | Whole blood. | Serum. | Plasma. | Equal parts of plasma and leucocytic cream. |
| | *hrs.* | | | | |
| Undiluted. | 4 | + | + | + | + |
| 1 : 5 | 4 | + | + | + | − |
| 1 : 25 | 4 | − | + | + | − |
| 1 : 125 | 4 | − | − | − | − |
| 1 : 625 | 4 | − | − | − | − |
| Undiluted. | 8 | + | + | + | + |
| 1 : 5 | 8 | + | + | + | + |
| 1 : 25 | 8 | + | + | + | − |
| 1 : 125 | 8 | + | + | + | − |
| 1 : 625 | 8 | − | + | + | − |
| Undiluted. | 12 | + | | | + |
| 1 : 5 | 12 | + | | | + |
| 1 : 25 | 12 | + | | | + |
| 1 : 125 | 12 | + | | | − |
| 1 : 625 | 12 | + | | | − |
| Undiluted. | 24 | | | | + |
| 1 : 5 | 24 | | | | + |
| 1 : 25 | 24 | | | | + |
| 1 : 125 | 24 | | | | + |
| 1 : 625 | 24 | | | | + |

agulation occurred. Washed guinea pig leucocytes and plasma or serum mixtures were also tested.

The following observations were made (Tables IV and V). (1) Pneumococci multiply rapidly in both the serum and coagulated plasma of immune animals, in some instances growth being more rapid in the plasma than in the serum. (2) Plasma and cells mixed in equal amounts inhibit growth to the same degree as the original whole blood. (3) A mixture of equal quantities of plasma and leucocytic cream delays the development of the culture more than the original whole blood.

TABLE V.

*Pneumococcus Cultures in Hen Blood, Plasma, Sedimented Cells, and Ground Sedimented Cells.*

| Dilution of culture with which capillary was charged. | Incubation. | Microscopic examination. | | | |
|---|---|---|---|---|---|
| | | Whole blood.* | Plasma. | Sedimented cells.† | Ground sedimented cells. |
| | *hrs.* | | | | |
| 1 : 10 | 11 | + | + | − | + |
| 1 : 100 | 11 | + | + | − | + |
| 1 : 1,000 | 11 | − | + | − | + |
| 1 : 5,000 | 11 | − | + | − | + |
| 1 : 10,000 | 11 | − | − | − | − |
| 1 : 10 | 28 | + | + | − | + |
| 1 : 100 | 28 | + | + | − | + |
| 1 : 1,000 | 28 | + | + | − | + |
| 1 : 5,000 | 28 | + | + | − | + |
| 1 : 10,000 | 28 | − | + | − | + |
| 1 : 10 | 58 | + | | + | |
| 1 : 100 | 58 | + | | − | |
| 1 : 1,000 | 58 | + | | − | |
| 1 : 5,000 | 58 | + | | − | |
| 1 : 10,000 | 58 | + | | − | |
| 1 : 10 | 72 | | | + | |
| 1 : 100 | 72 | | | + | |
| 1 : 1,000 | 72 | | | + | |
| 1 : 5,000 | 72 | | | − | |
| 1 : 10,000 | 72 | | | − | |
| 1 : 10 | 96 | | | + | |
| 1 : 100 | 96 | | | + | |
| 1 : 1,000 | 96 | | | + | |
| 1 : 5,000 | 96 | | | + | |
| 1 : 10,000 | 96 | | | + | |

* The hen was given 10 cc. of antipneumococcus serum 30 minutes before the blood was collected.

† Cultures of the sedimented cells on rabbit blood agar were positive throughout the experiment.

(4) Plasma plus washed leucocytes delays growth. (5) Immune serum and leucocytes do not retard growth to the same degree as plasma and leucocytes. (6) The compact cells[13] of a highly immune blood produce a prolonged latent period in the cultures which do not become positive on microscopic examination until after from 48 to 72 hours. (7) If the cells, as described under (6), are thoroughly ground with a mortar and pestle in fine, sterile sand, the pneumococci multiply as rapidly as in the serum or plasma. This is also the case when leucocytic cream or leucocytes are used in making the mixtures.

Here again it is indicated that the presence of leucocytes is essential to the retarding property of the blood, and since a mechanical destruction of the phagocytes, nothing being removed or added, robs the plasma-cell mixtures of their retarding property, it seems to be established that the act of phagocytosis plays a decisive part in the reaction. It remains to be explained, however, why a mixture of leucocytes and immune serum does not retard the development of the culture to the same extent as the plasma of the animal plus an equal number of leucocytes. A mechanical factor is involved here. In the serum-leucocyte mixtures the leucocytes quickly settle to the bottom of the capillary, leaving a layer of serum in which the pneumococci rapidly multiply. It should be stated here that when chicken plasma is prepared as described above the clot does not retract in the tubes but continues to occupy the whole tube, thus bringing the leucocytes into direct contact with the pneumococci which have been deposited on the walls.

### *Concentration of Antibodies and Retardation of Growth.*

*Experiment 6.*—A series of guinea pigs was given intravenous injections of varying quantities of antipneumococcus horse serum, the amount of serum injected ranging from 1 to 5 cc. 2 hours later capillary cultures were made of the whole blood of the guinea pigs. A normal guinea pig was included in the series. A set of the cultures was examined every 2 hours.

The latent period of the cultures was directly proportional to the quantity of serum injected, the cultures in the blood of the guinea pig which received 1 cc. of serum having a longer incubation period than those in normal blood, while the cultures made in the blood of the animal which received 5 cc. of serum were the last to develop (Table VI).

[13] The cells were sedimented by centrifugalization, but sufficient serum to opsonize the bacteria still adhered to the cells.

TABLE VI.

*Pneumococcus Cultures in the Blood of Passively Immunized Guinea Pigs.**

| Dilution of culture with which capillary was charged. | Incubation. | Microscopic examination. | | |
|---|---|---|---|---|
| | | Guinea Pig A (1 cc. of immune serum). | Guinea Pig B (5 cc. of immune serum). | Guinea Pig C (normal control). |
| | *hrs.* | | | |
| Undiluted. | 4 | + | − | + |
| 1 : 5 | 4 | − | − | + |
| 1 : 25 | 4 | − | − | + |
| 1 : 125 | 4 | − | − | − |
| 1 : 625 | 4 | − | − | − |
| Undiluted. | 8 | + | + | + |
| 1 : 5 | 8 | + | − | + |
| 1 : 25 | 8 | + | − | + |
| 1 : 125 | 8 | + | − | + |
| 1 : 625 | 8 | − | − | + |

* The antipneumococcus serum was given intravenously 2 hours before the cultures were made.

DISCUSSION.

The observations outlined above indicate that the whole blood of immune animals merely retards the multiplication of pneumococci instead of killing them, as is claimed by Wright and Heist and his coworkers. It is clearly demonstrated that failure to develop within 24 hours to the point of being positive on microscopic examination does not prove that the organisms with which the cultures were seeded have been killed. We have observed the development of cultures after a latent period of 72 hours. The authors referred to above ended their experiments after an incubation period of 24 hours. Should the organisms never multiply in the original medium, it could not be concluded that they had been killed, unless the cultures were proved to be sterile by failure of growth when transferred to a highly favorable medium. Even in this instance, particularly after a prolonged original incubation period, the possibility of involution would not be eliminated. It is claimed by Heist and his collaborators that a negative microscopic examination after 24 hours incubation invariably means pneumococcidal effect. The sterility test employed, however, was that of blowing the contents of the capillary tubes into broth.

The small number of organisms thus transferred was probably unable to overcome the resistance of the relatively large volume of liquid medium. In our experiments fresh rabbit blood agar tubes were substituted for the broth.

In every instance so far observed the degree of retardation of growth of pneumococci exerted by the whole blood corresponded to the degree of resistance of the corresponding animal to infection by these organisms. The converse, *i.e.* the absence of power in the blood to retard growth, is probably not always a true index of the susceptibility of the animal yielding it to infection. This seems to be true in the case of the cat, particularly on intravenous inoculation.

The whole blood reaction, as pointed out by Heist and his coworkers,[6] makes it possible to detect immunity responses which are not made evident by other methods. Only rabbits were used in the experiments on this point and our conclusions are correspondingly limited. We were unable to detect differences among untreated individuals of the same species. Hence it is improbable that the reaction will enable the detection of pneumonia susceptibility among human individuals, as the Schick test does for diphtheria.

The mechanism of the reaction is of particular interest, especially in connection with the light that its explanation may shed upon the nature of the factors and processes of pneumococcus immunity. Wright[5] designated the phenomenon as the "phagocyto-bactericidal power" of the blood, believing that the phagocytes were concerned in the reaction since plasma did not manifest it; but since defibrinated blood was inactive in this respect, he concluded that the chemical changes associated with the process of coagulation of the blood were essential factors in killing the pneumococci. The present work indicates that opsonization and phagocytosis are the essential agents, since the pneumococcidal reaction does not occur in the absence of either factor. It was shown, moreover, that with either factor constant the reaction varied quantitatively with the quantity of the second factor. It is conceivable that this relation will not be maintained when immune serum exceeds the concentration necessary for complete opsonization of all the organisms, the phagocytic capacity remaining constant. On the other hand, complete opsonization being maintained, there should be no limit to the number of organisms which

an increasing number of phagocytes could hold in check. This relation is similar to that which exists between immune serum and pneumococci in mouse protection experiments. In the latter instance, a certain quantity of antipneumococcus serum (0.2 cc.) protects against a maximum number (0.1 cc. of culture) of organisms, but when the number of organisms is increased, 0.2 cc. or larger quantities of the serum do not protect. This fact indicates that some essential factor (phagocytic capacity?) which is not increased has reached the limit of its power and the reaction fails. To carry the analogy further, the functioning time of the phagocytes in the capillary cultures is limited, and at the expiration of the period of phagocytic activity the reaction ceases. The pneumococci are now free to multiply regardless of the fact that immune bodies are still present. In the mouse protection tests a concentration of immune serum sufficient for complete opsonization continues for a limited time only. If the infection is so heavy that the phagocytes, functioning to the limit of their capacity, cannot destroy all the pneumococci during the opsonization period, some of the organisms escape destruction and are free to grow as soon as the opsonizing factor is eliminated. It has been shown that if the immune serum is kept at a sufficient concentration in the tissues of the infected animals by repeated injections, thus giving the phagocytes a longer working period, all the organisms are destroyed and the animal recovers.[14] This effect is not subject to duplication *in vitro* since the phagocytes which cease functioning because of death cannot be replaced.

It has not been definitely determined whether the retardation of growth of the culture is due merely to an inhibition of multiplication or to an actual destruction of a portion of the organisms, thus prolonging the incubation period. Indications are, however, that the action is one of inhibition only, since none of the cultures became sterile during the course of the experiment. It will be necessary to do further work before the matter is definitely cleared up.

[14] Bull, C. G., *J. Exp. Med.*, 1915, xxii, 466.

SUMMARY.

1. It has been shown that the whole uncoagulated blood of immune animals is not as highly pneumococcidal *in vitro*, as has been claimed by others.

2. Cultures of pneumococci in the fresh whole blood of immune animals, as compared with cultures in the blood of susceptible animals, show a greatly prolonged latent period, and, in a general way, the relative lengths of the latent periods of the cultures correspond to the relative resistances of the animals to infection by these organisms.

3. The blood of animals artificially immunized, both actively and passively, retards the growth of pneumococci in the same manner as the blood of naturally immune animals.

4. Microscopic examination of cultures of pneumococci in immune blood reveals chain formation, growth in clumps, and phagocytosis of the organisms by the polynuclear cells. It also shows that growth occurs first in the free serum, the clot being invaded later.

5. The retardation of multiplication depends on two factors, opsonization of the pneumococci by the immune serum and phagocytosis of the organisms by the polynuclear cells; growth readily occurs when either agent is absent.

6. Pneumococci multiply in defibrinated immune blood because few phagocytes are present after defibrination.

7. Pneumococci grow in the most potent immune blood after mechanical destruction of the white cells.

8. It has not been shown that immune blood does not kill a certain number of the pneumococci with which it is inoculated, but the tentative conclusion has been arrived at that no killing occurs since none of the tests became sterile during the course of our experiments.

# EXPERIMENTS ON THE PRODUCTION OF SPECIFIC ANTISERA FOR INFECTIONS OF UNKNOWN CAUSE.

## III. The Effects of a Serum Precipitin on Animals of the Species Furnishing the Precipitinogen.

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It has been shown previously that specific antisera for infections of which the exciting agent is unknown can be produced in some instances by the use of infected tissue itself as antigen; and that when such sera have been detoxified they can be used successfully for therapeutic purposes.[1] The toxicity is due to the presence of antibodies elicited by the tissue component of the antigen, which, needless to say, are highly injurious to animals of the species furnishing said antigen. It is easy to remove the more obvious of these injurious elements—hemolysins and hemagglutinins—by their selective absorption with red corpuscles, as was done in our type experiments. The precipitins we then encountered were weak and without recognizable action on the animal organism. Whether strong precipitins can cause damage has remained for determination, as has the point of how such damage could be avoided. The problem is not without practical significance in its relation to the utilization of the serum of infected human individuals as antigen.

The study of anaphylaxis has led to the development of a large and complex literature on the effects of the union of precipitin and precipitinogen upon an animal organism to which both are foreign; and a number of papers have been published on the results of injecting precipitinogen into animals which have developed a circulating precipitin. But there exist few observations, and these more or less casual, regarding the effects of a precipitin upon animals against

[1] Rous, P., Robertson, O. H., and Oliver, J., *J. Exp. Med.*, 1919, xxix, 283.

whose serum it is specifically effective. This alone concerns us here. Uhlenhuth and Haendel[2] state in a foot-note to other matters that guinea pigs show severe, anaphylaxis-like symptoms after the intraperitoneal injection of 0.5 to 1 cc. of an anti-guinea-pig rabbit serum with a precipitin titer of 1 to 20,000. The same serum in amounts of 0.75 to 2 cc. caused death. They give a few protocols suggesting tolerance to a second injection. Doerr and Moldovan[3] made closer observations. They state that the precipitating anti-guinea-pig rabbit serum which they employed had *in vitro* a slight hemolytic activity for guinea pig cells, no more than that of normal rabbit serum, yet 2 cc. intravenously killed guinea pigs within 2 hours, while 1 cc. gave rise to severe symptoms, and 0.5 cc. to dyspnea and slight symptoms. Following an intraperitoneal injection there was heightened resistance to a second injection 24 hours later. The experiments cited are few; and the authors make no mention of the presence or absence of hemagglutinins, nor, it seems, did they look for evidence of *in vitro* hemolysis. These are important points for the interpretation of their work, as will be shown.

### *Removal of Hemolysins and Hemagglutinins.*

It is well known that immunization with blood serum as antigen leads to the development not only of precipitins but of hemolysins and hemagglutinins, even though care has been taken to render the serum free of formed elements. In our first experiments along the lines of the work just described it became evident that these antibodies were seriously to be reckoned with. Hemolysis was never noteworthy on *in vitro* tests, being practically absent when guinea pig complement was employed, and in this regard our sera corresponded with the serum of Doerr and Moldovan. Yet these same sera, inactivated, gave rise *intra vasam* to a profuse breaking down of red cells, as evidenced by hemoglobinuria, hemoglobinemia, extreme anemia, spodogenous spleen, and free blood pigment in the fluid of the body cavities. Some fatalities were manifestly attributable to this cell destruction combined with hemagglutination. The latter phenomenon was always pronounced both *in vitro* and *in vivo*. That death can be due to it alone is well recognized. The blood of the animals sometimes showed an almost massive clumping. In view of all these findings the fact that the precipitating sera gave rise, as they did, to the more or less sudden death of guinea pigs was not surprising.

[2] Uhlenhuth and Haendel, *Z. Immunitätsforsch., Orig.*, 1909–10, iv, 761.

[3] Doerr, R., and Moldovan, J., *Z. Immunitätsforsch., Orig.*, 1910, vii, 223.

In order to free the sera of hemolysins and hemagglutinins resort was now had to selective absorption with guinea pig red cells. Our technique for this has been described in a previous paper.[1] Care was taken to keep the serum sterile and to free it of all possible stroma fragments by prolonged centrifugation prior to injection. Also, it was overabsorbed, that is, exposed to far more guinea pig red cells than were enough to remove the demonstrable antibodies for these elements. Our experience,[1] like that of others, has been that the strong hemolytic and hemagglutinative serum resulting from immunization with red cells is completely deprived of toxicity when thus treated. Such was far from being the case with the precipitating serum now in question. After absorption it retained the major part of its toxicity, giving rise to sudden death almost as often, in almost the same dose, and with the same symptoms as when untreated save for inactivation. But there was the difference that all lesions referable to hemolysis and hemagglutination were now lacking. Thus a number of late fatalities were avoided. Manifestly, from these results, Doerr and Moldovan were correct in supposing that the toxicity of their serum was due to another element than hemolysis.

*Removal of the Precipitin Does Not Remove Toxicity.*

What is this other element? The guinea pig cells used in the absorptions were freed of serum by careful and repeated washing in "gelatin-Locke's" solution, which keeps these usually fragile cells intact. The rabbit serum repeatedly incubated with several successive portions of them remained clear and almost free from hemoglobin. Friedemann[4] has shown that mixtures of red cells and hemolysin in the presence of complement may yield a toxic body before any hemolysis occurs. But this does not happen when complement is absent, as was regularly the case in our work. The possible influence of traces of the washing solution can be ruled out on the basis of previous experience. It seemed likely that the precipitin content of the serum, which was not lessened by repeated absorptions, constituted the toxic element. And in line with this idea, though not necessarily evidence for it, was the fact that the toxicity of different serum specimens

[4] Friedemann, U., *Z. Immunitätsforsch., Orig.*, 1909, ii, 591.

varied in general with their precipitin titer. Attempts were made, therefore, to detoxify the sera by the removal of their precipitin content through specific precipitation. The precipitin was readily removed. But to our great surprise the sera remained as toxic as before.

### *Specimen Experiments.*

*Experiment 1.*—Two rabbits which had received five intraperitoneal injections of guinea pig serum at intervals of 6 days were bled to death from the heart 10 days after the last, and the serum was at once pooled, inactivated, and tested for hemolysin and hemagglutinin. The undiluted serum caused a faint trace of hemolysis when incubated for 2 hours with equal parts of a 5 per cent suspension of guinea pig red cells and a 1 in 10 dilution of fresh guinea pig serum. In such mixtures agglutination was massive with quarter strength serum and was faintly seen with a 1 in 32 dilution of it. The injection of 2 cc. of the serum into the ear vein of a 200 gm. guinea pig[5] was followed in a few minutes by sneezing, restlessness, severe dyspnea, and complete prostration, with slow recovery during the next 12 hours.[6] The urine for some hours after the injection contained much hemoglobin.

50 cc. of the inactivated serum was now incubated under aseptic conditions with four successive portions of guinea pig red cells, twice washed in a large excess of gelatin-Locke's solution. The portions consisted of 7.5, 7.5, 9, and 6 cc. of packed cells respectively, and the period of incubation ranged from ¾ to 3 hours. No agglutination was observable in the last two serum-cell mixtures, and *in vitro* tests showed the complete absence of hemolysin. The absorbed serum, when injected into two guinea pigs of 225 and 215 gm. weight, in amounts of 2 and 1.35 cc. respectively, gave rise to exactly the same symptoms as the unabsorbed, though they were somewhat less severe. The urines of both animals remained free from hemoglobin.

The precipitin titer of the serum was now taken in mixtures of a constant amount of antibody with decreasing antigen, so as to avoid solution of the precipitate in an excess of the latter. By the use of a blood-counting pipette, previously standardized with mercury, as a measuring chamber, small amounts of the undiluted sera were mixed as such. Precipitation occurred in mixtures up

[5] For the technique of such injections see Rous, P., *J. Exp. Med.*, 1918, xxvii, 459. The operation is rendered more simple and certain if the ear is fixed on a ground glass platform, instead of the opaque one previously described, and transillumination is employed.

[6] 3 to 4 cc. of normal rabbit serum can be injected into the circulation of a 200 to 250 gm. guinea pig without the production of symptoms (see Friedberger, E., *Med. Klin.*, 1910, vi, 510).

to and including that containing 5,120 parts of rabbit serum to 1 from the guinea pig, and in 20,000 to 1 when the antigen was diluted to a constant bulk with salt solution after the usual method. The greatest precipitate, coarsely floccular, was seen at approximately 80 to 1 of the whole sera. Accordingly the bulk of the remaining rabbit serum was mixed with sterile guinea pig serum in this proportion, incubated 2 hours, left in the cold over night, and centrifuged until free from the several cubic centimeters of precipitate. It now failed to cause any clouding in mixtures with guinea pig serum above 16 to 1, yielded a slight cloud at 8 to 1, a dubious trace at 4 to 1, and none at 1 to 1. Yet this same serum, injected intravenously into three guinea pigs of 225, 205, and 225 gm. in amounts of 2.2, 1.45, and 1.35 cc. respectively, killed the first animal in 3 hours and 8 minutes, the second in 5½ hours, and in the third gave rise to a moderate "shock" with symptoms resembling those of anaphylaxis.

The results suggest that the serum was rendered, if anything, more toxic by the repeated absorptions.

It is known that a slight, slow precipitation takes place in pooled precipitin sera from different individuals of the same species; and Friedberger[7] has shown that precipitating mixtures will, in the presence of complement, yield a toxic product *in vitro*. To rule out this possible factor in the results, our tests were repeated with individual rabbit serum; and when several sera were to be pooled they were often subjected beforehand to a separate inactivation. In neither case was any difference noted in the results. In the experiment which now follows the sera were inactivated immediately after pooling, and subjected to precipitation with guinea pig serum prior to the absorptions with red cells.

*Experiment 2.*—The sera of four precipitin rabbits were pooled, inactivated, the precipitin titer was taken, whole guinea pig serum being used, and on the basis of the findings most of the pooled serum was submitted to an optimum precipitation. This was all done on the same day, as rapidly as possible. The optimum precipitation occurred in a 90 to 1 mixture with undiluted guinea pig serum, but clouding was noted in mixtures up to and including 5,120 to 1. The untreated serum injected intravenously into two guinea pigs of 325 and 400 gm., in amounts of 1.65 and 1.8 cc. respectively, killed the first mentioned animal within ¾ hour and caused great, though brief, prostration of the second. 1.85 cc. of the treated serum killed a 275 gm. guinea pig in 1 hour, and 2 cc. caused moderate symptoms in a guinea pig of 375 gm. All four animals had hemoglobinuria.

[7] Friedberger, E., *Z. Immunitätsforsch., Orig.*, 1909–10, iv, 636.

Both the treated and untreated sera were now submitted to five successive absorptions with twice washed red cells in the proportion of 23 cc. of serum to 2.3, 2.3, 2.3, 4.05, and 3.8 cc. of packed red cells. The contact periods ranged from 1 to 2 hours. Agglutination, which in the first mixture was well marked, diminished to a trace in the last one—an exactly similar trace for both the "precipitated" and untreated serum, as *in vitro* tests showed. No hemolysin could be found in the test-tube, with guinea pig complement. 1 part of salt solution was now added to 90 of the unprecipitated specimen, and comparative intravenous injections were carried out with it and with the precipitated serum (Table I).

TABLE I.

| Serum absorbed only. | | | Serum absorbed and precipitated. | | |
|---|---|---|---|---|---|
| Weight of animal. | Amount injected. | Result. | Weight of animal. | Amount injected. | Result. |
| *gm.* | *cc.* | | *gm.* | *cc.* | |
| 275 | 2.1 | Died in 1 hr., 51 min. | 250 | 2.1 | No symptoms. |
| 275 | 2.0 | " " 1 " 5 " | 275 | 1.8 | " " |
| | | | 275 | 2.2 | Very severe shock. |

Tests showed that the absorbed and precipitated serum still contained enough precipitin to cause clouding in mixtures up to and including 320 to 1 with whole guinea pig serum. An optimum was found at 20 to 1 and the serum submitted to a new precipitation in this proportion. Thereafter it still gave a moderate flocculation with an equal amount of guinea pig serum, but no clouding in mixtures above 10 to 1, or 16 to 1 when the precipitinogen was diluted with salt solution. Comparative tests *in vivo* were again made. 1 part of salt solution was added to 20 parts of the unprecipitated serum prior to the injections (Table II).

TABLE II.

| Serum absorbed only. | | | Serum absorbed and precipitated. | | |
|---|---|---|---|---|---|
| Weight of animal. | Amount injected. | Result. | Weight of animal. | Amount injected. | Result. |
| *gm.* | *cc.* | | *gm.* | *cc.* | |
| 375 | 2.0 | Moderate shock. | | | |
| 300 | 1.8 | Severe " | | | |
| 300 | 2.15 | Died in 11 min. | 300 | 2.2 | No symptoms. |
| 275 | 2.2 | Severe shock. | 250 | 2.2 | Very severe shock. |

At every stage in the treatment of the serum, cultures on agar and in bouillon were taken. These remained uniformly sterile.

In this instance, in contrast with Experiment 1, the attempts to detoxify the serum seemed to have some degree of success, and we were encouraged to further trials. These will not be detailed. They showed that individual differences in the test animals were mainly accountable for the wide variations in the results. Some animals were practically unaffected by the serum that killed others; and no matter how thoroughly the serum was exhausted with red cells and freed of precipitin it remained highly injurious. This was true even when it was given locally. For when injected subcutaneously or into the skeletal muscle it produced a severe lesion. But before describing this its general effects will be taken up.

*Effects of the Serum Deprived of Precipitin.*

In guinea pigs reacting to an intravenous injection of the exhausted and precipitated—or, for that matter, unprecipitated—serum there is a latent period of from 3 to 10 minutes during which the behavior is normal. Then the animal becomes restless, running about, scratching itself, perhaps sneezing, springing into the air, or twitching. The hair roughens, the urine and feces are usually voided, and an inspiratory dyspnea rapidly appears, accompanied in severe instances by cyanosis and complete prostration, followed by death in a few minutes or hours. Occasionally convulsions precede the fatal issue. Often there is only a paresis of the hind legs, or the animal is now prostrate, now on its feet again, and in these instances of milder symptoms recovery may be very rapid. More often, while recovering, the guinea pig sits crouched, cold, and with staring coat for some hours. When handled it is passive and weak. But by the next day recovery seems complete, and further observation proves that it indeed is so.

The animals that succumb show little on gross examination. The lungs may be distended, and the blood fail more or less markedly to clot, as in anaphylaxis; but these are by no means constant findings. There may be fresh petechiæ in the lungs and intestinal mucosa, as so often after violent death of any sort. This is all that is found if the animal has died within a few minutes of the injection. When it has survived for some hours the liver is always greatly congested, and in it there may be observed microscopically the only lesion that is

present with any regularity; namely, an acute central congestion of the lobuli, often with small hemorrhages, and, if death be deferred long enough, areas of necrosis as a result of the latter. There are no gross hemorrhages in the hepatic tissue. It would seem that the lesion might be secondary to an acute stasis in the heart or lungs, were not an abnormal distension of the right side of the heart entirely lacking at autopsy. In animals surviving several hours polymorphonuclear leucocytes may collect in considerable numbers in the pulmonary capillaries, but in those dying early this feature is absent. The blood shows no hemagglutination and its serum no trace of hemolysis.

The local lesion that follows a subcutaneous injection of 0.5 to 2 cc. of the serum, treated or untreated, is an edema, widespread about the point of injection, with numerous capillary hemorrhages and small, scattered foci of acute inflammation, which, however, do not go on to purulence. The injury may extend into the muscle. A broad, edematous, red-purple patch beneath the skin, with yellowish, ill defined, little, opaque patches and points scattered here and there throughout it, is characteristic. The acute inflammation is at its height at the end of 48 hours, but resolution occurs rapidly thereafter. Cultures remain sterile throughout. Necrosis such as the serum of cattle causes in guinea pigs[8] is never noted. The ordinary hemolytic and hemagglutinative serum obtained by the repeated injection of washed guinea pig corpuscles into rabbits, causes in guinea pigs a lesion somewhat similar in the gross to the one with which we are concerned; but there is this important difference, that the extravasated red cells which render the patch purple are for the most part hemolyzed so that few are seen on section, and those few are agglutinated into clumps. Furthermore, we have found, as already stated, that absorption with red cells rids such a serum of its ability to cause local lesions other than such slight ones as normal rabbit serum may mechanically produce.

[8] Uhlenhuth and Haendel, *Z. Immunitätsforsch., Orig.*, 1909, iii, 284.

*Parallel Experiments on the Dog.*

The work was now repeated on dogs, since for practical purposes it was important to learn whether our findings were peculiar to guinea pigs. The serum employed came from a number of rabbits repeatedly injected with dog serum. The precipitin titer was not so high as in the case of anti-guinea-pig serum, and hemolysins and agglutinins were no stronger, yet per kilo of animal it proved nearly as toxic. The toxicity was unimpaired by absorption with four portions of red cells followed by a single, unusually successful precipitation which removed practically all the precipitin. The intravenous injection of only 2.7 cc. per kilo of the sterile, exhausted and precipitated, or unprecipitated, serum regularly caused marked symptoms in dogs, and resulted once in death after 18 hours. A latent period of 5 to 10 minutes always followed the injection, and then the animal showed sudden signs of weakness, staggering and lying down, or standing unsteadily with drooping head. Vomiting and defecation took place, and soon there was complete prostration, the dog lying on its side in a semiconscious condition. The respiration was not disturbed, but there was an enormous drop in blood pressure, so that the pulse could no longer be felt and sometimes the heart beat was scarcely palpable. In most cases prostration did not endure more than 1 hour, after which recovery was rapid and complete. The animal which succumbed had received absorbed but unprecipitated serum. The single lesion found was similar to that observed in guinea pigs but more pronounced, being a destructive hemorrhagic congestion of the central half of the liver lobules. The blood in the large vessels, which failed to clot, showed no trace of hemagglutination, and the right side of the heart was not greatly distended. The hemorrhages into the liver substance were far more numerous and pronounced than in guinea pigs and this was the case as well with the lesions resulting from local injection in dogs, which otherwise did not differ from those in guinea pigs, save that edema was sometimes less marked. The numerous fine capillary hemorrhages in the edematous tissue at the site of injection often coalesced to give the appearance of a gross extravasate. Normal rabbit serum, old and new, in the amounts here dealt with, failed to cause any local or general disturbances.

*Relation of the Phenomena to Anaphylaxis.*

The "shock" produced by the serum will be seen from our description to resemble strikingly in both guinea pigs and dogs that called anaphylactic; but on close analysis points of difference declare themselves. At autopsy the lungs of guinea pigs may not be found distended, though they often are so; and the blood may be clotted. The latent period after an intravenous injection is much longer in dogs than that preceding the anaphylactic paroxysm. More important is the fact that desensitization cannot be effected either by small, graduated injections or by one that results in shock. We have given especial attention to this point, since Doerr and Moldovan present a few protocols which seem to indicate that they succeeded in desensitizing with their precipitating serum, and if this were the case it would offer a way to the safe, therapeutic utilization of our own. But their results must be referable to individual animal variation such as has already been mentioned, for our many tests have definitely shown that even after an injection of absorbed, or absorbed and precipitated, serum which calls forth a severe reaction, there may be no tolerance whatever to a second dose, whether it be given into the blood stream or locally. This is true in dogs as well as in guinea pigs. For example, a dog weighing 4 kilos was given 11.6 cc. of exhausted and precipitated serum into an ear vein. There resulted severe "shock," but with rapid and apparently complete recovery. 4 days later another and similar injection was given, and this called forth exactly the same severe but transient reaction. The animal was killed 6 days after the second injection, and in its liver active repair was found to be taking place of a recent hemorrhagic lesion such as has already been described. When several, small, desensitizing doses or one large one were used (in guinea pigs) the results were no better. The local effect in the guinea pig has some similarity to the Arthus phenomenon, but the latter is in our experience a less severe type of lesion in this species and far more difficult to elicit, at least with horse serum, while it lacks the hemorrhages caused by the absorbed and precipitated rabbit serum.

It is well known that the development of serum sickness is accompanied in man by the appearance of precipitins in the blood, and

that the urine may show a coincident albuminuria. We have followed the urines of a number of dogs and guinea pigs subjected to severe "shock" by the intravenous injection of precipitated or unprecipitated serum which had been exhausted with red cells. None showed noteworthy urinary change. Casts were regularly absent, and the slight trace of albumin occasionally noted was no greater than was inconstantly present prior to the injection.

Despite all this, there is no denying that the effects of the serum may be due to the same toxic principle or principles concerned in anaphylaxis. But, if so, an important difference in the quantitative relations must be assumed.

*Possible Sources of the Toxicity.*

Is the toxic element primarily present in the serum, or is it engendered by treatment? We feel convinced that the former is the case. The "shock" produced by the absorbed, or absorbed and precipitated, serum differs in no essential from that caused by inactivated but otherwise untreated precipitating serum, as observed by Uhlenhuth and Haendel, Doerr and Moldovan, and ourselves. Were it due to hemolysins and hemagglutinins persisting after absorption, the examination of the blood and the autopsy findings would give evidence of this, while furthermore the process of repeated absorption, even if incomplete, would greatly diminish the toxicity of the serum. Neither is the case. Our serum, as already mentioned, was overabsorbed; that is, submitted to more red cells than were necessary to take out all demonstrable hemolysins and agglutinins, a process which renders completely innocuous the ordinary hemolytic and hemagglutinative serum.

Many observations were made which bore on the question of whether gross precipitation within the animal body might not be a cause of the disturbances noted. Always the precipitated serum failed *in vitro* to cause a clouding when mixed with even a little more than its bulk of guinea pig serum, far less than the preponderant quantity encountered on its injection into the blood. Such tests would seem to rule out actual precipitation as the cause of disturbance, unless indeed the conditions with plasma differ greatly from

those with serum—and there is no reason to suppose that they do, since complement is unnecessary for the precipitin reaction. We have said that the toxicity of the different sera varied in general with their original precipitin titer. Yet that the "shock" engendered by the serum had no essential dependence on the immediate precipitin content was well shown in the results with serum from which the precipitin had been removed. The possibility remains that the toxic element may be a product of the interaction of precipitin and precipitinogen, one formed as readily when the two are brought together without as within the animal body. Against this is the fact shown by Friedberger[7] that specific precipitation *in vitro* fails to give rise to a toxic element unless complement be present; for it was absent in our experiments. But Friedberger made his tests of toxicity on animals of a species to which both precipitin and precipitinogen were strange, whereas in our work the precipitin was directed against the serum of animals of the species used for the tests, and just such serum was employed for the *in vitro* removal of precipitin.

There remains the interesting possibility of the presence in the serum of a hitherto unrecognized toxic antibody. Further work alone can justify any speculation in this direction.

## SUMMARY.

There is present in serum of high precipitin titer, produced by the repeated injection of rabbits with the blood-free serum of guinea pigs or dogs, a principle highly toxic for animals of the species furnishing the antigen. Intravenously the serum causes severe shock, and even sudden death, while locally it gives rise to acute inflammatory changes and profuse capillary hemorrhages. The complete removal of hemolysins and hemagglutinins from the serum by exposing it repeatedly to washed red cells lessens its toxicity to only a slight degree and one obviously dependent on these elements; while the further removal of precipitin by specific precipitation *in vitro* has no detoxifying effect whatever. Whether the toxic principle is a hitherto unrecognized antibody or perhaps a toxic product of the interaction of precipitin and precipitinogen,—one formed as readily in the test-tube as in the animal body,—remains to be determined.

The symptoms of guinea pigs and dogs given an intravenous injection of treated or untreated serum markedly resemble those of anaphylaxis, but our attempts at desensitization have been unsuccessful. The local lesion in guinea pigs is more severe than that of the Arthus phenomenon. But these differences from anaphylaxis may, of course, be dependent merely on differing proportions of constituents that are themselves, as yet, scarcely apprehended.

Our observations, as here summed up, were made with a practical point in mind, and as regards this point they are of a discouraging nature. In papers already published it has been shown that sera specifically effective against infections of which the excitant is unknown can in some cases be obtained by using infected tissue itself as antigen. Such sera must, of course, be deprived of antibodies injurious to tissue, prior to their employment in the animal body; and this was successfully accomplished in our early experiments by exhaustion with washed red cells. The purpose of the present work was to determine whether serum used as antigen gives rise to injurious principles in the antiserum. For the serum of infected individuals would in many diseases form a convenient antigen. It is evident that injurious principles result from its use, and that they are not removed from the antiserum when the latter is exhausted with red cells and its precipitin removed by specific precipitation, nor can their action be nullified by desensitization as carried out in anaphylaxis. Unless the obstacle of their presence is in some way overcome the body fluids of infected human beings cannot be practically utilized for the production of antiserum. In test animals the difficulty is not so grave. For we have found that the toxic antiserum produces no enduring lesions when it is administered intravenously in non-lethal doses.

# DETERIORATION OF CRYSTALLINE STROPHANTHIN IN AQUEOUS SOLUTION.

## Its Relation to Hydrogen Ion Concentration and a Method for Its Prevention.

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For clinical use crystalline strophanthin is commonly dissolved in normal salt solution or water and marketed in glass ampules. Sterilization is accomplished by autoclaving after the ampules have been filled and sealed. In making biologic assays of several lots of a commercial preparation of ouabain (g-strophanthin) by the cat method of Hatcher and Brody[1] wide variations in potency were found. On adding a drop of indicator, phenol red, to the contents of the ampules showing low potency, it was observed that they were decidedly alkaline in reaction, whereas freshly prepared, aqueous solutions of the drug were neutral or slightly acid. Experiments were undertaken to ascertain the cause of the deterioration in relation to the altered hydrogen ion concentration and to devise a method for preparing a stable solution for therapeutic purposes.

### *The Drug.*

Great variability in commercial preparations of crystalline strophanthin has been noted by Rowe,[2] who tested a number of lots bought on the open market by the "1 hour" frog method. The least active sample was 67 per cent as active as the standard (U. S. P.), whereas the most active was 240 per cent of the same standard, or about 3.5

[1] Hatcher, R. A., and Brody, J. G., The biological standardization of drugs, *Am. J. Pharm.*, 1910, lxxxii, 360. The cat unit represents the minimum lethal dose per kilo of cat, expressed in milligrams.

[2] Rowe, L. W., The variability of strophanthin, with particular reference to ouabain, *J. Am. Pharm. Assn.*, 1916, v, 1183.

times as potent. As indicated by Rowe, such variability in the crystalline product can, in large part, be attributed to the difficulty of obtaining unmixed seed of a desired variety, for there are more than twenty species of *Strophanthus* seed on the market and a given lot almost invariably contains specimens of several varieties. Further, different samples of the crystalline product contain variable amounts of water of crystallization; this, too, makes for differences in biologic activity expressed in terms of dry weight.

The drug employed in the present observations, as well as in a series of clinical studies to be reported later, was isolated from seeds purchased as *Strophanthus hispidus*, but apparently actually *Strophanthus gratus*. The procedure of isolation followed was based on the methods of Heffter and Sachs, and Thoms.[3]

The ground seeds were freed from fat with petroleum ether and the dried residue was extracted with cold 70 per cent alcohol. The extract was concentrated to a thick mass *in vacuo*, taken up in hot water, treated with hot basic lead acetate until no further precipitate formed, and filtered. The excess of lead was precipitated from the filtrate by means of a little ammonium sulfate and filtered off. The filtrate was saturated with ammonium sulfate, which precipitated a mixture of the amorphous and the crystalline strophanthin (ouabain), the last portions of the latter separating in crystalline form. The precipitate was dissolved in warm water, filtered, and the filtrate chilled and seeded with a few strophanthin crystals. After being allowed to stand in the ice box, the crystalline strophanthin was filtered off and recrystallized from water.

The crystals so obtained consisted of transparent, glistening platelets, containing 8.5 molecules of water of crystallization. The color reactions corresponded to those found by Arnaud and by Thoms.[4]

Physical constants:—Anhydrous substance: $[\alpha]_D^{20} = -30.2°$

Calculated for $C_{30}H_{46}O_{12}$: C, 60.17; H, 7.75

Found: C, 60.05; H, 7.81

Melting point (uncorrected): Softens: above 185°C; melts to paste: 187–188°C.; evolves gas: 195–197°C.

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[3] For the isolation and identification of the drug we are indebted to Dr. W. A. Jacobs and Dr. M. Heidelberger, of The Rockefeller Institute. They have also kindly furnished us with notes of the methods employed by them in these procedures. See also Heffter, A., and Sachs, F., Vergleichende Untersuchungen über Strophanthus-Glucoside, *Biochem. Z.*, 1912, xl, 83; Thoms, H., Die Strophanthus-Frage vom chemischen Standpunkt, *Ber. pharm. Ges.*, 1904, xiv, 104.

[4] Thoms, H., *Ber. pharm. Ges.*, 1904, xiv, 104.

Biologic standardizations of this crystalline strophanthin were carried out on thirty-one cats, and in twenty-six of the animals electrocardiograms were made at frequent intervals during the course of the experiments in order to determine the physiological effects of the glucoside upon the cat's heart. The average cat unit was found to be 0.104 mg. per kilo, a figure in accord with the standard of 0.10 mg. per kilo established by Hatcher and Brody. The electrocardiographic findings confirmed in their essentials the observations of Levine.[5] Chemically, biologically, and physiologically, therefore, the strophanthin at our disposal possessed those properties ascribed to the crystalline product obtained from *Strophanthus gratus* and usually marketed under the name "ouabain."

*Experiments with Glassware.*

Doubly distilled water, pH 6.0, was autoclaved for 20 minutes at 15 pounds pressure in various types of glass bottles and flasks, chosen at random from the laboratory supply. Immediately after autoclaving, the reaction of the water in the cheaper and softer varieties of container had become distinctly more alkaline, the pH ranging from 6.3 to 9.0+.[6] In the hard glass flasks (Pyrex) no significant alteration in reaction (less than 0.1) occurred. A standard 0.02 M phosphate mixture, pH 7.0,[7] autoclaved in the bottles giving off the maximum amount of alkali in the process of sterilization, showed no alteration in hydrogen ion concentration, the buffer action being adequate to compensate for the dissolved alkali.

A similar experiment was done with sixteen types of glass ampules obtained from a number of pharmaceutical firms and used by them in marketing their products. The ampules were filled with distilled water, sealed, and autoclaved. In every instance the water in the

[5] Levine, S. A., The action of strophanthin on the living cat's heart, *J. Exp. Med.*, 1919, xxix, 485.

[6] Determinations of hydrogen ion concentration were done colorimetrically. In the more alkaline solutions the results obtained by the colorimetric method were checked by the use of the hydrogen electrode.

[7] The standard phosphate mixtures used were prepared according to the directions of Sörensen (Sörensen, S. P. L., *Biochem. Z.*, 1909, xxi, 201; *Ergebn. Physiol.*, 1912, xii, 393).

ampules showed a change in pH, which now ranged from 6.2 to 9.0+. In order to titrate back to neutrality (pH 7.0) the most alkaline solution in the series, with phenol red as indicator, 2.6 cc. of 0.02 N hydrochloric acid per 100 cc. were required (Table I).

TABLE I.

*Hydrogen Ion Concentration of Distilled Water after Autoclaving in Various Types of Glass Ampules. Initial pH=6.0.*

| Ampule No. | pH of contained water after autoclaving 20 min. | Amount of 0.02 N HCl required to titrate 100 cc. of water to pH 7.0.* |
|---|---|---|
| | | *cc.* |
| 1 | 6.7 | |
| 2 | 6.2 | |
| 3 | 7.0 | |
| 4 | 7.0 | |
| 5 | 7.0 | |
| 6 | 7.0 | |
| 7 | 7.0 | |
| 8 | 9.0+ | 1.10 |
| 9 | 7.0 | |
| 10 | 7.2 | |
| 11 | 7.0 | |
| 12 | 9.0+ | 2.17 |
| 13 | 7.0 | |
| 14 | 9.0+ | 1.35 |
| 15 | 9.0+ | 2.10 |
| 16 | 9.0+ | 2.60 |

* Phenol red was used as indicator.

*Effect of Changes in Hydrogen Ion Concentration on Aqueous Solutions of Crystalline Strophanthin.*

A 2 per cent solution of strophanthin was made in standard 0.05 M phosphate mixtures with pH 5.0, 7.0, and 8.6. Biologically tested, the cat unit of these solutions was found to be 0.107 mg. per kilo,[8] their optical rotation − 0.97°. After autoclaving for 20 minutes at

[8] In making determinations of cat unit values at least three animals were employed to calculate the biologic activity. Each figure given, therefore, represents the average of three or more cat experiments. Where it is stated that no alteration in activity was noted, it is meant that less than a 10 per cent variation occurred, this variation representing changes in the third decimal place only.

15 pounds pressure in Pyrex flasks, no alteration in optical activity and no significant change in potency were observed in the acid or neutral solutions. The alkaline mixture, however, now had an optical rotation of − 0.93° and a cat unit value of 0.152. In short, when strophanthin was autoclaved in alkaline solution (pH 8.6), the molecule was partially decomposed, with resultant alteration in its ability to rotate polarized light and significant reduction in biologic activity (Table II).

The degree of alkalinity attained by aqueous solutions autoclaved in soft glass ampules is, therefore, more than ample to cause chemical changes in their contents.

TABLE II.

*Effect on Biologic Activity and Optical Rotation of Autoclaving Crystalline Strophanthin in 2 Per Cent Aqueous Solution at Different Hydrogen Ion Concentrations.*

| pH of 0.05 M phosphate solution. | Cat unit value per kilo. | | Optical rotation. | |
|---|---|---|---|---|
| | Before autoclaving. | After autoclaving. | Before autoclaving. | After autoclaving. |
| | *mg.* | *mg.* | | |
| 5.0 | 0.107 | 0.104 | −0.97° | −0.97° |
| 7.0 | 0.107 | 0.105 | −0.97° | −0.97° |
| 8.6 | 0.107 | 0.152 | −0.97° | −0.93° |

For clinical use it is convenient to employ crystalline strophanthin in dilute concentration, usually 0.01 per cent. Such solutions autoclaved in soft glass ampules, which gave off enough alkali to alter the reaction of the contents from pH 6.0 to pH 9.0, became at once biologically inert, more than four times the calculated lethal dose having no appreciable effect on the cat's heart. The contents of hard glass ampules, with no significant alteration in pH after sterilization (0.1 or less), retained full potency. In hard glass (Pyrex) such autoclaved solutions have been kept without deterioration for over 5 months.

Holste[9] noted an alkaline reaction in solutions of Boehringer's amorphous k-strophanthin which had deteriorated, and suggested that alkali given off from the glass containers might have induced hydrolytic cleavage of the glucoside.

[9] Holste, A., Zur Strophanthinfrage, *Z. exp. Path. u. Therap.*, 1918, xix, 153.

Like Thoms,[10] who observed marked reduction in toxicity for mice of sterile aqueous solutions of amorphous k-strophanthin, he found crystalline g-strophanthin to be more stable. Holste's observations, however, were carried no further, and no method for preventing deterioration was offered.

We have had made for our use tablets of g-strophanthin, 0.5 mg., triturated with sugar of milk. The amount of drug contained is so small and the percentage error in measuring out the powder for the individual tablet is, therefore, relatively so large, that this form of preparation was discarded as unsatisfactory.

### *A Method for Preparing Crystalline Strophanthin in Solution for Clinical Use.*

It is clear that stability of reaction is essential if the potency of strophanthin in solution is to be preserved. The glass commonly used in the manufacture of ampules for commercial purposes is, in large part, of the soft variety, yielding considerable amounts of alkali to solutions contained in it. It is advisable, therefore, to put up solutions of crystalline strophanthin in hard glass containers. Ampules of Pyrex glass have proved satisfactory. In order to avoid even slight changes toward the alkaline side, the drug may best be prepared in 0.02 M standard phosphate solution at pH 7.0.[11] This concentration of phosphate is harmless when injected intravenously in small amounts (5 to 10 cc.) into patients and does not interfere with either the chemical or physiological properties of the glucoside. Crystalline strophanthin in such a solution, in 0.01 per cent concentration (5 cc. contain 0.5 mg.), sealed in ampules, and autoclaved, has been preserved without evidence of reduction in biologic activity for more than 5 months.

[10] Thoms, H., Alte und neue Aufgaben der pharmazeutischen Chemie und insbesondere über die biologische Prüfung der Arzneimittel, *Ber. pharm. Ges.*, 1913, xxiii, 452.

[11] A 0.02 M phosphate solution at pH 7.0 contains 1.03 gm. of $KH_2PO_4$ and 1.76 gm. of $Na_2HPO_4$ (anhydrous) per liter.

SUMMARY.

1. Many of the glass containers commonly used in the laboratory, and most of the glass ampules employed in marketing sterile solutions for hypodermic or intravenous medication, yield sufficient alkali on autoclaving, to change the reaction of distilled water from pH 6.0 to pH 9.0.

2. This increase in alkalinity is sufficient to render biologically inert and partially to decompose aqueous solutions of crystalline strophanthin in the concentration ordinarily employed in the clinic.

3. It is suggested that for clinical use crystalline strophanthin be dissolved in 0.02 M standard phosphate solution at pH 7.0, and marketed in hard glass ampules, thereby insuring stability of reaction with preservation of biologic activity.

4. It should be borne in mind, both by laboratory worker and pharmacist, that the alkali yielded, on heating, by soft glass containers may be responsible for a considerable alteration in the hydrogen ion concentration of their contents.

# GIANT CENTROSPHERES IN DEGENERATING MESENCHYME CELLS OF TISSUE CULTURES.

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PLATES 16 AND 17.

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## INTRODUCTION.

In a previous communication (Lewis) on degeneration granules and vacuoles in living fibroblasts of chick embryos cultivated *in vitro*, it was noted that these granules and vacuoles, as they increase in number, tend to accumulate about the centriole which is located near one side or one end of the nucleus. Parallel with the accumulation important changes occur in this region of the cell. A clear area, gradually increasing in size, may develop about the centriole. This area, the centrosphere, may become as large or larger than the nucleus and is usually almost entirely free from degeneration granules and vacuoles. The latter form a corona about it, varying in width in different cells according to the number of granules and vacuoles which have accumulated.

Attention was also called to the fact that the mitochondria usually become arranged, more or less radially, about the centriole and the centrosphere, and that they may lie partly in the cytoplasm between the granules and vacuoles and partly in the clear peripheral region which is free from granules and vacuoles. In cells in which no distinct radial arrangement of the mitochondria about the centrosphere was seen and in which the mitochondria were usually in the form of short rods and granules, it was almost always noticed that the mitochondria in the cytoplasmic strands between the vacuoles were more abundant about the centrosphere than about the nucleus.

It is about the centriole or centrosphere, not about the nucleus, that the granules and vacuoles and the mitochondria are orientated.

In the above type of degeneration vacuolation of the cytoplasm is the predominant feature, but in the type considered in the present paper the enlargement of the centrosphere is the most characteristic change. The two forms of degeneration are not sharply separated from one another, and all gradations between them are found in the mesenchyme cells. We are uncertain whether these variations in the behavior of the cells during degeneration are dependent upon a difference in the type of mesenchyme cell or upon differences in the individual cells themselves, combined with differences in the cultural environment. In cultures of the same region sometimes one type predominates and sometimes the other. The two types may be characterized as follows:

*Vacuolar Type* (*Fig. 1*).—(1) Extensive vacuolation about the centrosphere and finally of the entire cytoplasm. (2) Enlarged centrosphere, usually without a sharp border or medullary zone. (3) Radial arrangement of mitochondria about the centrosphere.

*Giant Centrosphere Type* (*Figs. 3 to 8*).—(1) Slight or moderate vacuolation about the centrosphere. (2) Enlarged centrosphere with sharp border, centriole, and medullary and cortical zones. (3) Concentric or radial arrangement of mitochondria about the centrosphere.

The term centrosphere is used to designate the peculiar differentiated region about the centriole. It corresponds to the centrosphere or central body, the aster or astrosphere, of the dividing egg and cell. The terminology of this whole central apparatus is in a chaotic state and I have tried to select names that will not confuse. The centriole, when present, seems to be always in the form of a granule; it may appear to be single or double, usually the latter. Often the centrosphere consists of one or more zones which surround the centriole. These zones have been designated in various ways by different authors, but their significance and homologies are very obscure. The centrosphere in its most complete form, as seen in the fixed material, consists of a centriole, single or double, immediately surrounded by a clear medullary zone, and beyond this a large cortical zone. A granular zone, varying in thickness, may sometimes be seen between the medullary and cortical zones. This, however, is probably more or less transitory and accidental and is not to be considered as strictly

a part of the central apparatus. The cortical zone is surrounded by an outer granular zone, but this, also, is probably not strictly a part of the centrosphere. Both of the granular zones, inner and outer, consist, for the most part, of degeneration granules. The cortical zone may be sharply limited or may continue out into the cytoplasmic framework between the vacuoles. Its exact boundaries in this condition are difficult to define.

Distinct astral rays are not seen either in the living or in the fixed specimens, and they are also absent in the more normal dividing cells. Since in the fixed material the spindle fibers stand out clearly, it is probable that the astral ray fibril substance is either not present in these cells or is not laid down in such a manner as to appear, on coagulation, as radiating fibers. Often a more or less coarse radiation of the unvacuolated cytoplasm extends out from the centrosphere towards the periphery between the vacuoles, but it does not correspond to the astral rays.

The movements of the vacuoles and granules between centrosphere and periphery, or *vice versa*, have been described elsewhere in considerable detail (Lewis). The moving granules and vacuoles do not penetrate into the centrosphere but stop and become collected more or less at the periphery. This peculiar behavior of the granules would seem to indicate that the centrosphere is a semisolid gel or is surrounded by a membrane into which the granules cannot penetrate, while outside this is a semifluid in which granules and vacuoles move or are moved about by currents of cytoplasm.

Chambers concludes from his microdissection studies that "the development of the amphiaster is associated with the formation of two semisolid masses within the more fluid egg substance." Since these semisolid masses are probably centrospheres and correspond to those of the degenerating cells in the present study, Chambers' work lends support to the idea that the enlarged centrospheres of degenerating cells are also semisolid.

In the vacuolated type of cells it seems probable that the irregular cytoplasmic framework extending out from the centrosphere is also of a semisolid consistency and connects the semisolid centrosphere with a more or less semisolid peripheral layer, and that the vacuoles and granules occupy the more fluid part of the cytoplasm in which

are produced currents that carry the passive granules back and forth. Such currents might result from changes in osmotic conditions, due to various metabolic states. The mitochondria lie in the cytoplasmic framework between the vacuoles and are usually more or less radially arranged about the centrosphere. Their movements are different in character from those of the granules, as one would expect them to be on the theory that they lie in a more solid framework.

In the type of cells with enlarged centrospheres, an intervacuolar framework is not so apparent, but there is usually concentrated about the centrosphere a more deeply staining cytoplasm in which lie mitochondria and granules and vacuoles, while the more peripheral part of the cytoplasm stains very lightly or not at all.

*Normal Mesenchyme Cells in Tissue Cultures.*

In fixed and stained specimens of the first 20 hours most of the cells appear normal or nearly so. The centriole can often be recognized without difficulty. It is usually double and lies near the nucleus, at the side or at one end, more often at the side. It may be near the center of one side or towards either end. Often it lies within an indentation of the nucleus which occasionally is so deep that the double centriole appears to be within the nucleus. There is little or no radial arrangement of the mitochondria except such as usually occurs in multipolar cells in which the long mitochondria extend out into the processes. There is usually, however, more cytoplasm on the side of the nucleus which includes the centriole, and it also appears to be denser. There are always cells in a culture in which one cannot determine the centriole with absolute certainty, and others in which no structure corresponding to it can be made out. A few cells in cultures incubated for a period of 24 hours show the beginnings of a radial arrangement of the mitochondria and an increase in the number of fine granules about the centriole. The mitochondria are long, thread-like, and often branched. They never seem to exhibit any definite relation to the nucleus, and only occasionally, as mentioned above, to the centriole.

*Degenerating Mesenchyme Cells.*

The three cultures described below have been selected from among others in which the enlarged or giant centrosphere type of degeneration predominates. The first culture, No. 404, shows many transitions between more or less normal cells and those with enlarged centrospheres, while the second culture, No. 441, shows, for the most part, only cells with enlarged centrospheres. The degenerative processes have gone still further in No. 444, in which many of the centrospheres have lost their centrioles and medullary zones, and granules and vacuoles can be seen in the centrosphere itself.

*Culture 404 (Text-Figs. 1 to 9).*

This culture is from an explant of subcutaneous tissue and muscle of the leg of an 8 day chick embryo cultivated in Locke-Lewis solution (Locke's solution plus 0.25 per cent dextrose plus 10 to 20 per cent chicken bouillon). The growth was abundant. After 48 hours the culture was fixed in Zenker's fluid, without acetic acid. The preparation was stained lightly with iron-hematoxylin.

Most of the cells show signs of degeneration but there is little vacuolation of the cytoplasm, even where the centrosphere has become as large as the nucleus. Various stages in the enlargement and differentiation of the centrosphere are to be found associated with changes in the arrangement of the mitochondria and the accumulation of fine granules, probably degeneration granules, about the centriole and centrosphere. Degeneration seems, as a rule, to be more advanced near the periphery of the growth than near the explant.

In a sector of the outer two-thirds of the growth about one-third of the cells show large centrospheres, one-third small and medium sized ones, and in about one-third of the cells no centrospheres are found. The mesenchyme cells are mostly multipolar in character, and such as are usually found in cultures associated with the outgrowth of skeletal muscle.

In the earlier stages of degeneration there is no accumulation of special centrosphere material about the centriole. The fine degeneration granules gather more or less about the centriole and the mitochondria tend to become more or less radially arranged about it. As

Text-figs. 1 to 9, from Culture 404, show stages in the development of the centrosphere.

TEXT-FIG. 1. Accumulation of degeneration granules about the centriole.

TEXT-FIG. 2. Appearance of a clear medullary zone about the centriole with a corona of degeneration granules and radial mitochondria.

TEXT-FIG. 3. Beginning cortical zone formation; mitochondria separated from the granular zone.

TEXT-FIG. 4. The same condition except that the mitochondria are short and thick and concentrated more about the cortical zone.

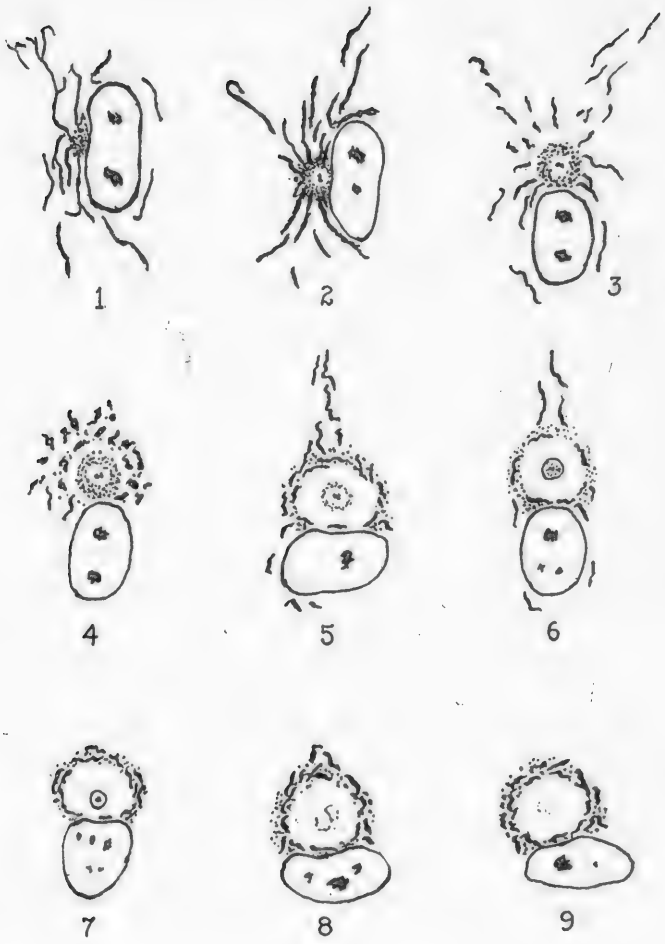
TEXT-FIG. 5. Well developed centrosphere with the centriole, medullary and cortical zones, inner granular zone, and degeneration granules about the cortical zone. The mitochondria are becoming concentric.

TEXT-FIG. 6. Inner granular zone gone. The medullary zone stains more deeply than the cortical zone.

TEXT-FIG. 7. The mitochondria are mostly concentric about the centrosphere; there is none about the nucleus.

TEXT-FIG. 8. The medullary zone is disintegrating and the centriole is not visible.

TEXT-FIG. 9. The medullary zone and centriole have disappeared.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the process goes on there gradually develops about the centriole an area free from degeneration granules, vacuoles, and mitochondria (Text-figs. 1 to 4). This clear area never becomes very large and is surrounded by a halo of very fine granules. It forms, I think, the medullary zone of the large complete centrosphere. The mitochondria, which are arranged more or less radially about this area, at first have their inner ends among the granules which surround it. There are many points about the clear medullary zone that are still obscure—whether it is fluid or solid, and what its origin is; or whether it is a centriole vacuole, pushing away the degeneration granules from the centriole as it enlarges. If it is a fluid it must have some sort of a limiting membrane. There is no special reason to suppose that it comes from the granules which at an earlier stage closely surround the centriole. It develops very much like a vacuole about a granule but in the living cell it never takes up the neutral red or other vacuolar dyes, such as methylene blue (Ehrlich) or brilliant cresyl blue. The granules which surround the periphery of the medullary zone obscure its outline more or less; later, when these granules disappear, as in Text-figs. 6 and 7, a sharp border or thin membrane separates it from the cortical zone. This membrane is not always homogeneous. The medullary zone is often eccentrically placed. In the early stages the medullary zone does not seem to take up the hematoxylin stain, but in later stages, after the granular zone has disappeared, the medullary zone is often stained darker than the cortical zone. In what I judge to be still older stages, the medullary zone becomes very obscure or may disappear altogether. Sometimes faint irregular traces of it are seen. The centriole also disappears in these more advanced stages or, at least, fails to stain, for no trace of it can be found (Text-figs. 8 and 9).

The details of the formation of the cortical zone are just as obscure as those of the medullary zone. There is a gradual accumulation of cortical substance about the medullary zone and the fine granular zone which immediately surrounds it. The mitochondria seem to be pushed away from the medullary zone (Text-figs. 3 and 4). Ultimately there is formed a large cortical zone which may give to the centrosphere a size about that of the nucleus (Text-figs. 5 to 9). The origin and nature of this cortical substance are uncertain. It

seems probable that it consists of the more or less hypothetical substance known as archoplasm, kinoplasm, spongioplasm, etc., and there is evidence that it has been drawn into the centrosphere from the surrounding cytoplasm.

In the early stages we have seen that the long mitochondria radiate from the centriole and centrosphere far out into the cytoplasm. Some significant changes take place, more or less parallel with the increase in size of the centrosphere. The mitochondria tend to accumulate more and more immediately about the enlarging centrosphere and the radial arrangement gradually gives place to a more or less concentric arrangement, as seen in Text-figs. 4 to 9. The mitochondria are probably not actively motile bodies but dependent upon movements of the cytoplasm. I picture the stages of the development which takes place somewhat in this order. There is a drawing or a flowing in of a certain part of the cytoplasm, presumably the archoplasm, to the centrosphere. This flowing in of the archoplasm in the earlier stages helps to explain the usual more or less radial arrangement of the mitochondria. They are pulled in, somewhat reluctantly as it were, towards the center, and thus tend to become extended out in radial lines. In the early stages of the process the cortical zone is without sharp boundary and extends out into the cytoplasm. There is evidently some sort of transformation of the archoplasm as it accumulates about the medullary zone. This may be merely a change to a more semisolid consistency or to a condition such as would stop the mitochondria at the periphery and also prevent the penetration of new granules and vacuoles which accumulate around it. Finally, most of the archoplasmic material becomes concentrated within the centrosphere or immediately about it, and the outlying cytoplasm appears to be clear and devoid of granules and mitochondria. The substance that is finally concentrated within the centrosphere is different from that immediately about it and from that in the more peripheral regions of the cell. The substance immediately about the centrosphere is also peculiar. It contains the mitochondria and granules and is itself finely granular and stains more deeply than the peripheral cytoplasm. These differences can be seen in the living, as well as in the fixed and stained material.

The granular zone is variable and probably consists merely of degeneration granules which in the living cell take the neutral red.

What might be called an inner granular zone lies between medullary and cortical zones; this is not constant and is probably not a part of the centrosphere proper.

In early stages, as we have noted, degeneration granules, which take up neutral red in the living cell, accumulate about the centriole. It seems probable that the developing medullary zone pushes them back from the centriole. The gradually enlarging medullary zone is always surrounded by a halo, composed at first of fine, later of coarse granules. As the cortical zone develops this halo diminishes. Some of the granules appear to be pushed to the periphery of the gradually enlarging cortical zone where other newly formed granules have been accumulating. A few scattered granules are often seen in the cortical zone. All conditions between a well marked inner granular zone and its complete absence can be found. In many cells the granules disappear ultimately, either dissolving or finding their way to the periphery of the cortical zone.

The mitochondria, which are at first long and thread-like and often branched, gradually break up into short threads, rods, and granules which, in turn, may swell up into vesicle and other polymorphic forms. In general the changes parallel those in the centrosphere, but not always, indicating that the changes are more or less independent of each other. It is obvious that the mitochondria accumulate about the enlarging centrosphere and also that the central ends of many are pushed back from their original position at the periphery of the medullary zone to the enlarging periphery of the cortical zone. The latter process would also tend to change their position from radial to concentric. Fig. 2 illustrates a stage in the concentration of the archoplasm about the centriole. This large, flat, mesothelial-like cell consists of a broad, scarcely stainable, peripheral layer of cytoplasm and a more central, finely granular area that appears to be closing in about the centrosphere, which consists of a centriole and small medullary zone.

*Culture 441 (Figs. 3 and 4).*

This culture is from an explant of a piece of a large blood vessel of a 6 day chick embryo in Locke-Lewis solution. The growth was abundant. It was fixed after 3 days in Zenker's fluid, without acetic

acid, and deeply stained in iron-hematoxylin. Most of the cells show large centrospheres that are smaller than the nucleus. Of 44 cells, carefully examined, 7 showed a centriole, medullary zone, and cortical zone; 10, medullary and cortical zones, the centriole being doubtful; 13, only medullary and cortical zones with no trace of a centriole; 14, only cortical zone material. A few of the cells showed vacuolation of the centrosphere. The centrospheres in these cells are more like those usually pictured as cancer cell inclusions. They have a light cortical zone and a deeply staining medullary zone. There are practically no transition stages, as shown in Culture 404.

The centrospheres are rather sharply outlined except where the surrounding denser part of the cytoplasm with its granules, vacuoles, and mitochondria obscures the edge. The material of the centrosphere is also different in character from the rest of the cytoplasm. The latter shows a moderate degree of vacuolation about the centrosphere. The mitochondria vary from rods and threads to granules and are more or less radially arranged about the centrosphere.

*Culture 444* (*Figs. 5 to 8*).

This culture is from an explant of subcutaneous tissue and muscle of an 8 day chick embryo in Locke-Lewis solution. The growth was abundant. It was fixed after 48 hours in Zenker's fluid, without acetic acid, plus a few drops of 2 per cent osmic acid. The preparation was deeply stained with iron-hematoxylin and counterstained with Bordeaux R.

Most of the cells in the culture show a small amount of vacuolation and a large centrosphere which has reacted in a characteristic manner to the fixation and staining, so that it has a different texture from the rest of the cell cytoplasm. Many of these centrospheres have a clear-cut margin which defines them from the surrounding vacuolated cytoplasm, but there is apparently no visible membrane about the centrosphere. In a count of 100 cells, in 8 the centrosphere was larger than the nucleus, in 18 about the same size, in 70 smaller than the nucleus, and in 4 cells a centrosphere was not recognizable. The medullary zone and centriole are either almost invisible or have disappeared entirely. Vacuoles in the centrosphere are frequent, and tend to obscure and confuse the picture. In only 4 of the 100 cells

carefully examined did the centrosphere seem to be entirely free from vacuoles. These are usually at or near the center of the centrosphere but not symmetrically placed as a rule. A vacuole within the centrosphere, with a granule lying near its center, is often difficult or impossible to differentiate from the medullary zone and its centriole. Most of the centrospheres also contain granules. These are usually smaller than, but sometimes as large as mitochondrial granules, and in fixed material, stained in the manner described, it is impossible to differentiate between them. In living material, stained with neutral red and Janus black No. 2, mitochondrial granules are rarely seen within the centrospheres. Granules which take up the neutral red, however, do occur, and it is probable that the granules in these centrospheres are of this type and are to be considered as degeneration products. There is the possibility that some of the granules may represent centrioles and that a multiplication of them has occurred, such as was found by Heidenhain in certain giant cells. The granules are usually most numerous near the center of the centrosphere and also in that part of it next to the nucleus, as seen in Fig. 8. Sometimes they are arranged in a ring around a central clear area, possibly the medullary zone (Fig. 7). The difficulty or impossibility of distinguishing the various types of granules in specimens stained with iron-hematoxylin should certainly guard one against too free speculation as to their true nature. It is only because I have examined hundreds of living cells doubly stained with neutral red and Janus black No. 2 or Janus green that I venture even to suggest the nature of the granules.

The vacuolation of the cytoplasm is, as a rule, of moderate degree only and is confined, for the most part, to the region immediately about the centrosphere. The thin clear peripheral part of the cytoplasm is usually entirely free from vacuoles as in Fig. 8. It is about the centrosphere rather than about the nucleus that the vacuoles are found, as most of the figures show. It is not uncommon for the vacuoles to extend entirely around the centrosphere, even between it and the nucleus. The nucleus is usually at one side or near one end of the cell while the greater mass of the cytoplasm, with its vacuoles and granules, surrounds the centrosphere. This is occasionally much emphasized as shown in Fig. 6 in which the nucleus is widely separated

from the centrosphere and its surrounding cytoplasm by an elongated narrow stalk. The nucleus has very little cytoplasm about it and no vacuoles or mitochondria.

In these preparations the degeneration granules are sometimes difficult or impossible to distinguish from the mitochondrial granules. Many of the granules, which are found among the vacuoles and around the centrosphere are, however, probably mitochondrial granules or small mitochondrial vesicles.

The mitochondria are usually concentrated about the centrospheres. The majority of the cells contain only the small granular type. Other cells show both the rod and granular forms (Fig. 5), and a few show threads, rods, and granules (Fig. 8). The transformation of the normal thread-like mitochondria into rods and granules usually more or less parallels the progressive degenerative changes (vacuolation of the cytoplasm and enlargement of the centrosphere), but this is not always the case, and the two processes are more or less independent of each other.

No two cells degenerate in exactly the same manner. Each cell in the culture possesses its own peculiar individual features by which it can be distinguished from any other cell, and the degenerative changes tend to emphasize these differences. In studying a culture of this character we find ourselves dealing with individuals whose peculiarities depend only partially upon environment, especially in cases where the cells are side by side. The environment may play an important part, however, in different regions of the same culture, for the conditions in the neighborhood of a cell located at the periphery of the growth vary markedly from those surrounding a cell near the explant. In cells which have approximately the same environment as, for instance, where they lie side by side, the individual differences between them must be due, for the most part, to differences of the cells themselves, such as the interrelations in size and space and the interactions between nucleus, cytoplasm, mitochondria, centriole, etc.

*Relation to Cancer Cell Inclusions.*

The enlarged centrospheres in these degenerating cells are of peculiar interest because of their similarity to certain of the cytoplasmic inclusions of cancer cells (Plimmer's bodies, bird's eye inclusions, cancer parasites, cancer cell inclusions, etc.).

20 years ago and more these peculiar bodies found in cancer cells attracted much attention because of the claim by Plimmer and others that they were the cancer parasites. Plimmer found these bodies in 1,130 out of 1,278 cases. Previously, Pianese had stated his belief that the cancer cell inclusions were due to secretions. Later Borrel advanced the theory that these peculiar cytoplasmic inclusions were similar to the centrosomes of normal cells. His attention was attracted by the similarity of the centrosome apparatus of the spermatocytes of the guinea pig to the structures in the cancer cells; and he considered the cancer cell inclusions as typical centrospheres. LeCount states:[1] "The points of similarity between these bodies and the archoplasmic structures of normal cells, a likeness that had been indicated by Borrel, lead me to stop further search in the regional metastatic growths for this 'cancer parasite.' The analogies are not simply those of morphology and position, but also of affinity for dyes." LeCount's description of the cancer bodies can be applied almost verbatim to the giant centrospheres in our cultures. There are certain minor differences, as can be seen by comparing figures, but the essentials are similar. This is likewise true with the figures of Borrel, of Pianese, and of Greenough. Concerning Plimmer's bodies, the last mentioned author concludes that "their appearance, staining reactions, and situation in the cell, are such as to justify the hypothesis that they are the result of the secretory activity of the epithelial cell."

Many of the "*Vaccinekörperschen*," as figured by Hückel, seem to be typical centrospheres, often with medullary and cortical zones and with or without a centriole.

It is well known that cancer cells are subject to degenerative changes such as occur in other tissues, and if it is true, as I believe, that the enlarged centrosphere in the mesenchyme cells of the present cultures is one of the expressions of cell degeneration, it is not surprising that similar bodies occur in both types of cells. The fact that an enlargement of the centrosphere occurs in the degeneration of embryonic cells suggests that the appearance of similar centrospheres in cancer is an indication that cancer cells are likewise embryonic in character and might be used in support of the theory that cancers arise from embryonal rests.

In cancer we are dealing with epithelial cells and it is somewhat surprising, at first glance, to find such an important phenomenon as the enlargement of the centrosphere common to such different types of cells as epithelium and embryonic mesenchyme. It is probable that the fundamental metabolic processes and the mechanism for them

[1] LeCount, p. 386.

are essentially similar. If my interpretation of Hückel's figures is correct, the enlargement of the centrosphere in degeneration is not confined to cancer or to embryonic cells and may be looked for in other pathological conditions of the body tissues. The fact that it occurs in the cells of tissue cultures where, to a certain extent at least, we can control the environment, brings within the realm of experimental solution the factors which cause this condition. The cultures in which it has been found were made in the usual manner and the factors involved in producing this type of degeneration, with an especially large and well defined centrosphere, are not known. All the cells in all the cultures, unless they are washed repeatedly in fresh media, degenerate and die in from 2 to 15 days, and it is not uncommon to find giant centrospheres in degenerating cultures regardless of the age of the culture.

*The Centrosphere.*

We are safe in assuming that these cells with vacuoles, granules, and enlarged centrospheres are not normal, and since they die sooner than the more normal cells, these changes may be looked upon as degeneration changes. The enlargement of the centrosphere is probably an active response to the peculiar environment of the cell. The factors concerned have not been analyzed but it will probably be possible to do so experimentally. The analogy between this particular enlargement of the centrosphere and that characteristic of cell division is suggestive, in that the causal factors are probably identical or nearly so. In the normal tissue cell division results and the metabolic balance is restored; in the abnormal cells other factors prevent this and death results. Embryonic cells are repeatedly passing through a cycle of growth and division determined by the metabolic processes which take place within the cell. The enlargement of the centrosphere is one phase of this.

The material composing the centrosphere gathers about the centriole as though the latter were the dynamic center of the cell, as suggested by Boveri. The origin of this material is uncertain. In the larger centrospheres it looks and behaves differently from the rest of the cytoplasm. Whether it is chemically or physically altered cytoplasm or a concentration of the so called archoplasm or altered archoplasmic material remains to be determined.

SUMMARY.

1. Degenerating mesenchyme cells in tissue cultures cultivated in various modifications of Locke's solution often have giant centrospheres.

2. These giant centrospheres develop gradually around the centriole and in their most complete condition consist of centriole, medullary zone, and cortical zone.

3. Preceding the appearance of the centrosphere the centriole is surrounded by degeneration granules or granules and vacuoles, and as the centrosphere develops these are forced first to the periphery of the medullary zone and later, when it develops, to the periphery of the cortical zone.

4. The mitochondria become orientated about the centriole and centrosphere, at first more or less radially, later concentrically. They change from threads to rods and granules.

5. The giant centrospheres and cancer cell inclusions (Plimmer's bodies, etc.) are identical.

BIBLIOGRAPHY.

Borrel, A., Les théories parasitaires du cancer, *Ann. Inst. Pasteur*, 1901, xv, 49.

Boveri, T., cited by Wilson, E. B., The cell in development and inheritance, New York and London, 2nd edition, 1900, 76.

Chambers, R., Jr., Microdissection studies. II. The cell aster: a reversible gelation phenomenon, *J. Exp. Zool.*, 1917, xxiii, 483.

Greenough, R. B., Cell inclusions in cancer and in non-cancerous tissue, *J. Med. Research*, 1902, vii, 360.

Hückel, A., Die Vaccinekörperschen, *Beitr. path. Anat. u. allg. Path., 2nd Suppl.*, 1898.

LeCount, E. R., The analogies between Plimmer's bodies and certain structures found normally in the cytoplasm, *J. Med. Research*, 1902, vii, 383.

Lewis, W. H., Degeneration granules and vacuoles in the fibroblasts of chick embryos cultivated *in vitro*, *Bull. Johns Hopkins Hosp.*, 1919, xxx, 81.

Pianese, G., Beitrag zur Histologie und Aetiologie des Carcinoms, *Beitr. path. Anat. u. allg. Path., 1st Suppl.*, 1896.

Plimmer, H. G., On the etiology and histology of cancer, with special reference to recent work on the subject, *Practitioner*, 1899, lxii, 430.

## EXPLANATION OF PLATES.

### PLATE 16.

Figs. 3 and 4 have been retouched.

FIG. 1. Vacuolar type of degeneration in mesenchyme cells. 3 day culture from the leg of a 7 day chick embryo. Culture 406. Zenker's fluid, without acetic acid, was used for fixation, iron-hematoxylin for staining. × 1,250.

FIG. 2. Concentration of the archoplasm (?) about a small centrosphere with a centriole in mesothelial-like cells of a 5 day culture from the leg of an 11 day chick embryo. Culture 440. Zenker's fluid, without acetic acid, was used for fixation, iron-hematoxylin for staining. × 550.

FIG. 3. Mesenchyme cell with a large centrosphere showing cortical and medullary zones; no centriole. Mitochondria—more or less radial threads, rods, and granules—are shown. A few vacuoles may be seen about the centrosphere. 3 day culture of mesenchyme from a large blood vessel of a 6 day chick embryo. Culture 441. Zenker's fluid, without acetic acid, was used for fixation, iron-hematoxylin for staining. × 1,250.

FIG. 4. Mesenchyme cells from the same culture (No. 441). The cell on the left has a large centrosphere showing cortical and medullary zones; no centriole; mitochondria, rods and granules about the centrosphere; vacuoles few. The cell on the right has a large irregular centrosphere of cortical substance only and somewhat vacuolated. It is surrounded by mitochondria and vacuoles. × 1,250.

### PLATE 17.

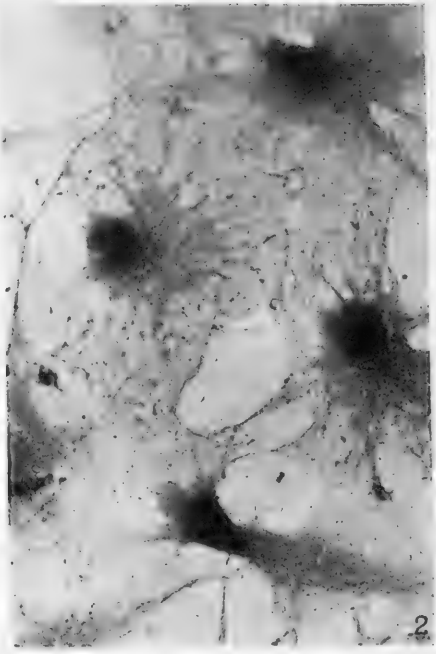
Figs. 5 to 8 have been retouched.

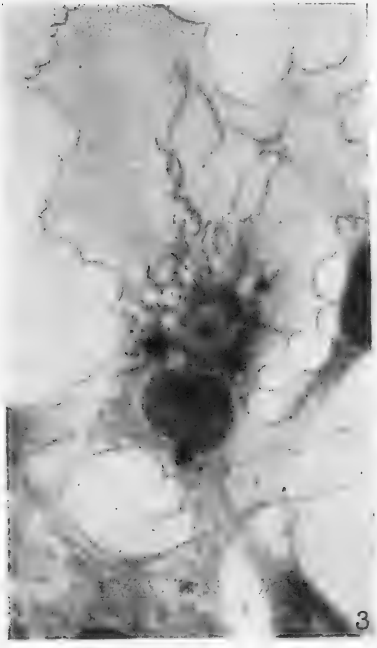
FIG. 5. Mesenchyme cells from Culture 444, 2 day culture from the leg of an 8 day chick embryo. The lower cell shows a large oval centrosphere with sharp outline containing vacuoles and granules; mitochondria—short threads and rods—more or less concentric; very few vacuoles. The upper cell shows a similar but smaller centrosphere surrounded by mitochondrial granules and few vacuoles. These centrospheres seem to consist of cortical substance only. Zenker's fluid, without acetic acid, plus a few drops of 2 per cent osmic acid was used for fixation, iron-hematoxylin and Bordeaux R for staining. × 1,250.

FIG. 6. Mesenchyme cells from Culture 444. The lower cell shows a large, well defined centrosphere. It appears to have a medullary zone surrounded by granules and a large cortical zone. Mitochondrial rods and granules surround the centrosphere. In the upper cell the nucleus is separated by a long, slender stalk from the body of the cell, in which is seen the centrosphere surrounded by mitochondrial granules and a few vacuoles. The centrosphere seems to consist of cortical substance only. × 1,250.

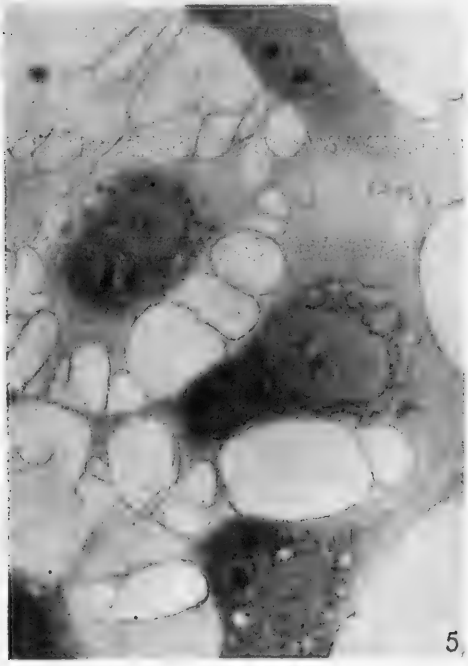
FIG. 7. Mesenchyme cells from Culture 444. The upper cell shows a large centrosphere. The medullary zone with a centriole surrounded by granules is seen. The cortical zone contains scattered granules and is surrounded by granular mitochondria and vacuoles. The lower cell is smaller and contains a smaller nucleus and smaller centrosphere. × 1,250.

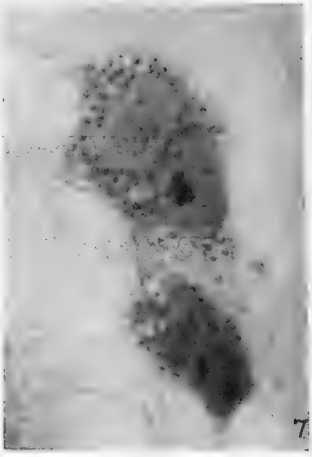
FIG. 8. Mesenchyme cell from Culture 444. The large centrosphere shows many vacuoles and granules; no medullary zone or centriole. The mitochondrial threads, rods, and granules are more scattered than usual. × 1,250.

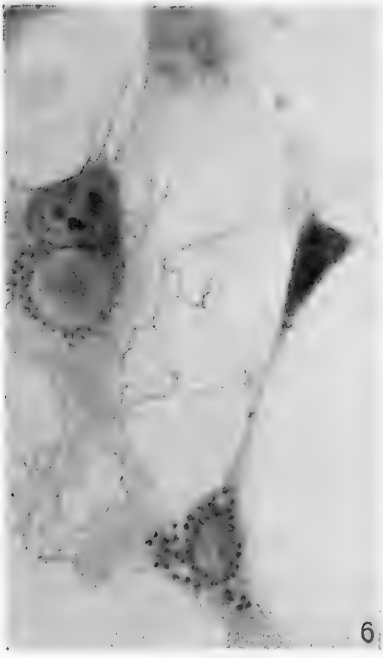


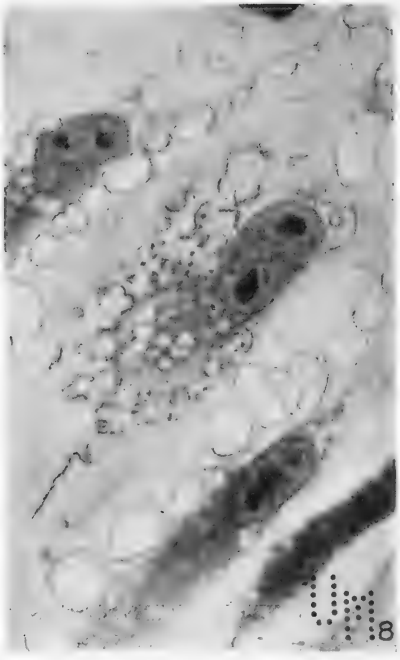



(Lewis: Tissue cultures.)




(Lewis: Tissue cultures.)

# THE ORMATION OF VACUOLES DUE TO BACILLUS TYPHOSUS IN THE CELLS OF TISSUE CULTURES OF THE INTESTINE OF THE CHICK EMBRYO.

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PLATES 18 AND 19.

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Lewis (1919, *b*) has shown that the formation of vacuoles in the cells of tissue culture is a phenomenon frequently associated with degeneration. It was not surprising, therefore, to find that the presence of bacteria in the culture resulted in a similar behavior of the cells. While several species of bacteria did not cause the vacuolation of the cells, others did bring about this reaction. None of these, however, caused the process to take place so rapidly or so extensively as did the typhoid bacillus.

## *Technique.*

The cultures of bacteria were grown on agar slants.[1] The inoculation was made by touching a platinum wire against the growth of organisms and then inserting it into the hanging drop of the tissue culture. The results of the experiments were extremely complicated and only one phase, the formation of vacuoles, will be discussed in the present paper.

Pieces of the intestine of chick embryos (7 to 9 days incubation) were explanted into Locke-Lewis solution (sodium chloride 0.9 per cent plus potassium chloride 0.042 per cent plus calcium chloride 0.025

[1] Transplants from the stock cultures of the Department of Pathology of the Johns Hopkins University were obtained through the kindness of Dr. L. D. Felton. All cultures of bacteria were prepared by Mr. D. T. Smith, who kindly tested out every culture of typhoid used in these experiments.

per cent plus sodium bicarbonate 0.02 per cent plus dextrose 0.25 per cent plus chicken bouillon 15 per cent) by means of the usual tissue culture technique. The growth of this particular series of cultures was among the best so far obtained in the laboratory.[2] Practically every culture grew luxuriantly. Not only was there abundant migration of connective tissue cells, but large membranes extended out from the endodermal lining of the intestine and also from the mesothelium. Frequently an extensive plexus of sympathetic nerve fibers was present in the new growth. These cultures, unlike those described by Lewis (1919, *b*), lived from 4 to 6 days without signs of degeneration, and only in the oldest cultures (8 to 10 days) did the number of vacuoles become marked.

The cultures of the intestine were carefully examined after 24 or 48 hours, not only in regard to the size and extent of the growth, but also as to the condition of the individual cells, especially concerning the presence of vacuoles. Any culture containing cells in which vacuoles had already appeared was discarded. This happened in only a few instances. After the preparation had been thus examined the cover-slip was removed and the hanging drop was inoculated with *Bacillus typhosus*. The number of organisms carried over was necessarily large in proportion to the amount of tissue. The culture was then resealed, turned upside down for a few seconds in order to distribute the organism, and returned to the warm box (39°C.). The controls were prepared in the same manner except that the platinum point, instead of carrying bacteria, was sterilized by passing it through the flame.

## RESULTS.

As shown by Table I, it is evident that the introduction of *Bacillus typhosus* into the hanging drop of the tissue cultures resulted in rapid vacuolation of the cells of the growth (Figs. 1 to 4). These vacuoles appeared as minute round bodies which rapidly grew larger and in certain instances fused together to form large vacuoles (Fig. 4). In the ordinary degeneration of the cultures, such as described by Lewis

[2] Dr. Mary J. Hogue kindly made for me a series of cultures of the intestine of the chick embryo.

(1919, *b*), not all the cells became vacuolated at the same time or to an equal extent, but in these infected cultures all the cells of the cultures suffered this change and all were in the same stage of vacuolation at the same time (Figs. 1 and 2). This phenomenon was delayed when the culture was bathed with fresh solution before inoculation. It was also delayed if, instead of touching the hanging drop with bacteria, one suspended the organisms in fresh medium and placed a drop of this upon the cultures. Vacuolation, however, took place within a short time after inoculation (10 to 12 hours), even in the fresh solution, regardless of the age of the culture, so that it seemed as though the typhoid bacilli acted in such a way as to hasten markedly some process which resulted in the vacuolation of the cell.

The vacuoles were usually collected at one side of the nucleus around a central mass of cytoplasm (Fig. 3) so as to suggest that this central part corresponds to the centrosphere described by Lewis (1919, *a*). The vacuoles frequently appeared to be free from granules or to contain only minute ones. In other instances one or more granules were present.

About an hour after inoculation one or two organisms were sometimes observed within a single vacuole in one of the cells of the growth. After a longer interval, not only did more vacuoles contain bacilli but the number of these to a single vacuole was greater (Fig. 3). The vacuoles were confined to the cytoplasm of the cell and neither vacuoles nor bacteria were observed within the nucleus. While the proportion of cells containing organisms was always small, such cells were not limited to any one type of tissue, appearing in the connective tissue as well as in the mesothelium and even in the endoderm from the lining of the intestine (Figs. 1 to 3). Jones and Rous carried out observations in regard to the ability of isolated connective tissue cells from either rat or chicken cultures to ingest *Staphylococcus pyogenes albus*. They claim that such isolated connective tissue cells rarely take up carmine granules and that the phagocytosis of the bacteria occurred only in the presence of serum (antistaphylococcus). No mention is made of vacuoles in these cells.

In almost all cases in which the inoculated cultures were kept for several hours the typhoid bacilli remained motile and increased in number. Smyth performed an extensive series of experiments in

TABLE I.

| Culture. | Bacteria. | Observations. | | | | |
|---|---|---|---|---|---|---|
| | | After 10 min. | After 30 min. | After 1 hr. | After 2 hrs. | Later observations. |
| 1. $I_s$ 48 hrs.* | 12 hr. culture of No. 80 B. | Several very small vacuoles appear in all cells of growth. Some mitotic figures. | Many vacuoles present in all cells. No mitotic figures. | Many vacuoles in all cells; larger and fewer in connective tissue cells than in other cells. | In 5 of the cells one vacuole in each contains bacteria. Vacuoles large and few in number in connective tissue. Many small vacuoles in each cell of endodermal membrane and of mesothelium. No mitosis. | Fixed and stained at end of 2½ hrs. (Figs. 3 and 4). |
| 2. $M_s$ 72 hrs. | 3 day culture of No. 80 B. | | Many vacuoles. | | | |
| 3. $M_s$ 96 hrs. | " " | Many vacuoles. Fixed and stained. | | | | |
| 4. $M_s$ 48 hrs. | " " | A few vacuoles in each cell. A number of mitotic figures. | A number of vacuoles. | | No more vacuoles than were present at end of half hour. | |
| 5. $I_s$ 96 hrs. | " " | A number of small vacuoles in each cell. | | | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| 6. $I_8$ 7 days. | 3 day culture of No. 80 B. | | A number of vacuoles. | Full of vacuoles. | | Culture dead after 20 hrs. |
| 7. $I_8$ 48 hrs. | 6 day culture of No. 80 B. | A few vacuoles in each cell. | | | Number of vacuoles not greatly increased. | |
| 8. " " | " " | | A few vacuoles in each cell. | | | At end of 4 hrs. a few small vacuoles in each cell. |
| 9. " " | No bacteria. | | No vacuoles in the cells. | | | At end of 4 days growth large and only occasional cell had vacuoles. |
| 10. $I_9$ 48 hrs. | 1 drop of 10 day culture of No. 80 B in Locke-Lewis solution. | | A very few small vacuoles in each cell. | | | At end of 20 hrs. all cells dead. Cytoplasm is composed largely of a network with here and there the walls of the vacuoles still present. |
| 11. " " | " " | | " " | | | " " |

* $I_8$ 48 hrs. indicates a culture of the intestine of an 8 day chick embryo, 48 hours after explantation; $M_8$ 72 hrs., a culture of the muscle of an 8 day chick embryo, 72 hours after explantation, etc. 80 B, etc., is the departmental number of the typhoid culture. Cultures 20 to 24 were bathed with a fresh solution before inoculation. Nos. 37 to 40 and 59 to 66 were washed with Locke-Lewis solution in which neutral red (1: 50,000) had been dissolved, and later inoculated. In Nos. 10 to 18 the organism was suspended in Locke-Lewis solution and a drop of this placed on the culture. Nos. 35 and 41 to 45 were stained with neutral red in Locke-Lewis solution some time after inoculation.

TABLE I—*Continued.*

| Culture. | Bacteria. | Observations. | | | | |
|---|---|---|---|---|---|---|
| | | After 10 min. | After 30 min. | After 1 hr. | After 2 hrs. | Later observations. |
| 12. $I_9$ 48 hrs. | 1 drop of 10 day culture of No. 80 B in Locke-Lewis solution. | | | A few vacuoles in the cells. | | At end of 20 hrs. all cells dead. Cytoplasm is composed largely of a network with here and there the walls of the vacuoles still present. |
| 13. " " | " " | | | " " | | " " |
| 14. " " | " " | | A very few small vacuoles in each cell. | | | " " |
| 15. " " | " " | | " " | | | " " |
| 16. " " | " " | | " " | | | " " |
| 17. " " | " " | | " " | | | " " |
| 18. " " | " " | | | A few vacuoles in the cells. | | " " |

| | | | | | | |
|---|---|---|---|---|---|---|
| 19. $I_9$ 48 hrs. | Drop of Locke-Lewis solution. | | | | No vacuoles in cells. | At end of 4 days good growth. No vacuoles. |
| 20. $1_5$ 72 hrs. Cultures washed with Locke-Lewis solution. | 18 hr. culture of No. 80 B M. | | A few vacuoles in each cell. | | No increase in vacuoles. Bacteria very motile. | At end of 20 hrs. partly dead. Cells full of vacuoles. Bacteria increased and motile. |
| 21. " " | " " | | " " | | " " | " " |
| 22. " " | " " | | " " | | " " | " " |
| 23. " " | " " | | " " | | " " | " " |
| 24. " " | No bacteria | | | | | At end of 20 hrs. good growth. Many mitotic figures. Very few vacuoles. |
| 25. $I_6$ 72 hrs. | 12 hr. culture of No. 80 B M | | Many vacuoles in all cells. | | | |
| 26. $I_8$ 48 hrs. | 24 hr. culture of No. 80 B M. | Small vacuoles in all cells. | Many vacuoles. Fixed [illegible] and stained. | | | |

TABLE I—*Continued.*

| Culture. | Bacteria. | Observations. | | | | |
|---|---|---|---|---|---|---|
| | | After 10 min. | After 30 min. | After 1 hr. | After 2 hrs. | Later observations. |
| 27. $I_8$ 48 hrs. | 24 hr. culture of No. 80 B M. | Small vacuoles in all cells. | Many vacuoles. | Fixed and stained. | | |
| 28. " " | " " | " " | " " | | Fixed and stained. | |
| 29. " " | " " | " " | " " | | | |
| 30. $I_8$ 72 hrs. | 60 hr. culture of No. 80 B M. | Few vacuoles. | | | A number of vacuoles in the cells. Clasmatocytes have taken up the bacteria. | At end of 3 hrs. fixed and stained. |
| 31. " " | Bacteria frequently long threads. | " " | | | " " | " " |
| 32. " " | " " | " " | | | " " | " " |
| 33. " " | " " | " " | | | " " | " " |
| 34. " " | " " | " " | | | " " | " " |

| | | | | | | |
|---|---|---|---|---|---|---|
| 35. $I_9$ 48 hrs. | 24 hr. culture of No. 80 B $M_2$. | Few vacuoles. | | | | At end of 4 hrs. stained with neutral red in Locke-Lewis solution. Many various shaped and sized red vacuoles, some containing one or more bacteria. Clasmatocytes have red vacuoles containing unstained bacteria. At end of 20 hrs. almost all growth dead. Some cells have unstained vacuoles containing active bacteria. A few cells had red vacuoles. |
| 36. " " (3 cultures). | No bacteria. | No " | | | | Lived 7 days. Good growth. Few vacuoles. |

TABLE I—*Continued.*

| Culture. | Bacteria. | Observations. | | | | |
|---|---|---|---|---|---|---|
| | | After 10 min. | After 30 min. | After 1 hr. | After 2 hrs. | Later observations. |
| 37. $I_8$ 48 hrs. Culture washed with Locke-Lewis solution + neutral red. | 24 hr. culture of No. 80 B M. | | Few vacuoles. | | Many red vacuoles. No bacteria in vacuoles even in clasmatocytes. | At end of 20 hrs. growth largely dead; diffuse red. Vacuoles unstained. A few cells still alive have many large red vacuoles. |
| 38. " " | " " | | " " | | " " | " " |
| 39. " " | 24 hr. culture of No. 80 B $S_2$. | | " " | | " " | |
| 40. " " | " " | | " " | | " " | |
| 41. $I_8$ 48 hrs. | " " | | | | | At end of 4 hrs. stained with neutral red in Locke-Lewis solution. A number of red vacuoles. Some red vacuoles have unstained bacteria in them. Some bacteria in red vacuoles motile; some outlined with red in the cell. |

| | | | | | | |
|---|---|---|---|---|---|---|
| 42. $I_3$ 48 hrs. | 24 hr. culture of No. 80 B $S_2$. | | | | | At end of 4 hrs. stained with neutral red in Locke-Lewis solution. A number of red vacuoles. Some red vacuoles have unstained bacteria in them. Some bacteria in red vacuoles motile; some outlined with red in the cell. |
| 43. $I_3$ 72 hrs. | " " | | | | | " " |
| 44. " " | " " | | | | | " " |
| 45. $I_3$ 48 hrs. | " " | | A number of vacuoles in each cell. | | Vacuoles with very motile bacteria in them. Many vacuoles in each cell. | At end of 3 hrs. stained with neutral red. A number of red vacuoles have quiet bacteria in them. Many red vacuoles in each cell. |
| 46. " " | No bacteria. | | | | | At end of 4 hrs. no vacuoles. Lived 7 days. At end of this time there were a number of vacuoles in some cells. |

TABLE I—*Continued.*

| Culture. | Bacteria. | Observations. | | | | |
|---|---|---|---|---|---|---|
| | | After 10 min. | After 30 min. | After 1 hr. | After 2 hrs. | Later observations. |
| 47. $I_7$ 48 hrs. | 24 hr. culture of No. 80 B $S_4$. | | A number of vacuoles. | | | At end of 20 hrs. cells almost all dead. Bacteria grew and are motile. |
| 48. " " | " " | | A few vacuoles in each cell. | No increase in vacuoles. | | At end of 20 hrs. good growth. Many vacuoles in each cell. Bacteria increased and are motile. |
| 49. " " | " " | | A number of vacuoles in each cell. | | | At end of 20 hrs. all dead. Bacteria grew and are motile. |
| 50. " " | No bacteria. | | | | An occasional vacuole. | At end of 20 hrs. good growth. Many figures of division. A few vacuoles in a few cells. |
| 51. " " | 24 hr. culture of No. 80 B $S_5$. | | A number of vacuoles. | Many vacuoles; some large. | | |
| 52. " " | " " | | A number of vacuoles. Some dead cells. | Many vacuoles; some large. Fixed and stained. | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| 53. $I_7$ 48 hrs. | 24 hr. culture of No. 80 B $S_8$. | | Many vacuoles. | Many vacuoles. Some large ones have one motile bacillus. | | At end of 20 hrs. few cells survive; all full of vacuoles. Only one vacuole seen containing a bacillus. |
| 54. " " | " " | No vacuoles. | | | No vacuoles. | At end of 20 hrs. no vacuoles. |
| 55. " " | 24 hr. culture of No. 80 C. | | Many vacuoles. Many dead cells. | Many vacuoles. No bacteria seen in vacuoles. | | At end of 20 hrs. all dead. Bacteria grew. |
| 56. " " | " " | | Many vacuoles. No dead cells. | | | At end of 20 hrs. a few spindle cells survive. These are full of vacuoles. Bacteria grew. |
| 57. " " | " " | | " " | | | Dead at end of 20 hrs. Bacteria grew. |
| 58. " " | " " | | Many vacuoles; some large. | | | " " |
| 59. " " Washed with neutral red in Locke-Lewis solution. | " " | A few small red vacuoles. | | A number of small red vacuoles. | | At end of 20 hrs. all cells dead. Bacteria grew and are motile. |
| 60. " " | " " | | | " " | | " " |

TABLE I—*Concluded.*

| Culture. | Bacteria. | Observations. | | | | |
|---|---|---|---|---|---|---|
| | | After 10 min. | After 30 min. | After 1 hr. | After 2 hrs. | Later observations. |
| 61. $I_7$ 48 hrs. Washed with neutral red in Locke-Lewis solution. | 24 hr. culture of No. 80 C. | | | | | At end of 4 hrs. many red vacuoles of different shapes, some containing an unstained granule. Dead at end of 20 hrs. |
| 62. " " | " " | | | A number of small red vacuoles. | | At end of 4 hrs. many cells dying. A number of unstained vacuoles. Many red vacuoles. No bacteria seen in vacuoles. At end of 20 hrs. cytoplasm of cells has become a network and is full of unstained vacuoles. Cell is diffuse red. Bacteria grew and are motile. |
| 63. " " | " " | | | " " | | " " |

| | | | | | | |
|---|---|---|---|---|---|---|
| 64. $I_7$ 48 hrs. Washed with neutral red in Locke-Lewis solution. | 48 hr. culture of No. 80 B $S_4$. | | | A number of small red vacuoles. | | At end of 20 hrs. a number of cells still alive; full of bright red vacuoles. Bacteria motile. |
| 65. " " | " " | | | " " | | Dead at end of 20 hrs. Bacteria motile. |
| 66. " " | " " | | | " " | | At end of 20 hrs. some of growth still alive with cells full of large red vacuoles. |
| 67. $I_7$ 24 hrs. | 48 hr. culture of No. 80 B $S_5$. | | | Many vacuoles. | | |
| 68. " " | " " | | | " " | | |
| 69. " " | " " | | | " " | | |
| 70. " " | No bacteria. | | | No " | | At end of 20 hrs. good growth. Many mitotic figures. |

regard to the effect of bacteria upon plasma cultures. He states that *Bacillus typhosus* never grew in plasma alone. However, when the plasma had been incubated to destroy its bactericidal action, the bacteria developed freely with especial affinity for the tissue cells. According to Smyth the typhoid bacteria had no toxic action on the tissue cells. It is difficult to understand this statement in the light of the present experiments, as in every culture in which the organism grew, there resulted a rapid degeneration of the tissue. It is possible that the strain of typhoid bacillus employed by Smyth was different from that used in these experiments.

The normal cultures contained many cells in the process of division. When these cultures were inoculated, the cells undergoing mitosis, as well as the resting cells, became vacuolated. The dividing cells completed the process but no new mitotic figures appeared in the growth, except in rare instances. While the normal culture of the series usually exhibited eight to ten dividing cells, even when the culture was more than 4 days old, the infected cultures seldom showed any.

When the cultures were stained with neutral red before inoculation the vacuoles were red from their first appearance. This color faded upon the death of the cell. The vacuoles were stained at various times during their formation and always became bright red, regardless of whether they contained bacteria or not. The motility of the organism in the vacuoles usually decreased when the preparation was stained with neutral red.

These vacuoles cannot be regarded as the result of the ingestion of the bacteria, as they appeared very rapidly in all the cells at the same time, being small at first but later increasing in size and even fusing to form exceedingly large vacuoles. The bacteria were never found in the cells until some time after the vacuoles had appeared and then only in a few of the many hundred cells comprising the growth. Even when present they were not found in all the vacuoles of a cell but usually in only one. The organisms were very motile in the unstained vacuole, occasionally dashing from side to side, but more frequently circling rapidly round and round the boundary of the vacuole. If the vacuoles are the result of the ingestion of some substance by the cell, as has been claimed by some writers, one

would not expect to find any difference in results, whether the original hanging drop were used or had been replaced by a fresh one. However, the utilization of a fresh drop did markedly alter the results; so that, instead of vacuoles appearing at once and rapidly increasing in size, it was some time before they became noticeable in numbers. The results obtained in regard to phagocytosis will not be given here, as they are at present inseparable from many other complicated factors.

Metchnikoff, who not only performed many experiments himself but discussed the work of innumerable other investigators upon the subject of phagocytosis, gives only a meager description of vacuoles in cells other than protozoa. Metchnikoff and his followers seemed to take it for granted that the leucocyte contained digestive vacuoles; this, however, is not the case. Normal leucocytes do not contain vacuoles, although they rapidly become vacuolated under abnormal conditions. Plato[3] claims that there are no bodies that stain with neutral red in the leucocytes of the freshly drawn blood. Miss R. Prigosen in a number of observations upon the blood of the hen and also upon human blood in this laboratory found no vacuoles of any kind in leucocytes immediately upon their withdrawal from the body. Frequently writers describe the ingestion of bacteria without mentioning vacuoles, and other authors, even with the use of neutral red, claim that the ingested particles stain with neutral red, but they often either fail to describe the stained vacuoles or definitely state that the vacuoles remain unstained.

Barber inoculated *Nitella*, *Saprolegnia*, and *Achyla* with *B. typhosus*. In nearly very instance the organism grew luxuriantly in the cells. Barber does not mention vacuoles in the cytoplasm other than the vacuole which was a definite structure of the cell. He states that the bacteria apparently met with no resistance in passing from the cell vacuole into the protoplasmic layer. It was not possible to find any harmful action of the protoplasm upon the bacteria. The cell finally died as a result of parasitism.

A number of writers discussing necrosis, degeneration, or old age frequently describe vacuolation of the tissue. Calkins[4] mentions this in regard to paramecium. Dr. W. Gary told me of certain experiments on autolysis of protozoa in which vacuoles appeared in the cytoplasm of the organism. Miss Prigosen, in the studies previously mentioned, cites a number of cases in which lack of oxygen caused the formation of vacuoles in different sorts of cells. Symmers shows vacuolation of the cells in multiple non-inflammatory necrosis of the liver. Lewis (1919, *b*) describes the vacuolation of tissue cultures associated with degeneration of these growths.

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[3] Plato, p. 913.

[4] Calkins, p. 127.

Many substances have been introduced into the hanging drop of tissue cultures in an effort to obtain information as to what factor in the life history of the cell is chiefly concerned in the formation of vacuoles. Food substances such as egg albumin, blood serum, dextrose, glycogen, aleuronat, etc., when added to the medium did not cause a marked or rapid increase in the number of vacuoles. Some of these substances caused a slight increase in the number of neutral red granules. Other substances such as phosphorus, carbon dioxide, urea, and ammonia caused all sorts of distortions of the cells, and often death ensued, but they failed to result in a constant vacuolation of the cytoplasm. As far as has been determined the effect of the typhoid bacteria upon the culture seems to be one that sets up some disturbance of the normal behavior of the cell in such a way as to result rapidly in a type of degeneration which is not uncommon in old cultures under usual conditions; *i.e.*, such a degeneration as has been described by Lewis (1919, *b*) in uninoculated cultures. It would be interesting to determine whether cloudy swelling common in acute infection is a form of vacuolation of the cells.

## CONCLUSION.

The introduction of *Bacillus typhosus* into the hanging drop of a tissue culture of the intestine of a chick embryo leads to the rapid vacuolation of the cells of the tissues which comprise the growth of the cultures. The cells of the connective tissue, the endodermal membrane, the mesothelium, and also the clasmatocytes exhibit the ability to ingest *Bacillus typhosus* in the cultures.

## BIBLIOGRAPHY.

Barber, M. A., The effect on the protoplasm of *Nitella* of various chemical substances and microorganisms introduced into the cavity of the living cell, *J. Infect. Dis.*, 1911, ix, 117.

Calkins, G. N., Protozoology, New York, 1909, 127.

Hogue, M. J., The effect of hypotonic and hypertonic solutions on fibroblasts of the embryonic chick heart *in vitro*, *J. Exp. Med.*, 1919, xxx, 617.

Jones, F. S., and Rous, P., The phagocytic power of connective tissue cells, *J. Exp. Med.*, 1917, xxv, 189.

Lewis, W. H., The behavior of the centriole and the centrosphere in the degenerating fibroblasts of tissue cultures, *Am. J. Physiol.*, 1919, *a*, xlix, 123.

Lewis, W. H., Degeneration granules and vacuoles in the fibroblasts of chick embryos cultivated *in vitro*, *Bull. Johns Hopkins Hosp.*, 1919, *b*, xxx, 81.

Metchnikoff, E., Immunity in infective disease, translation by Binnie, H. G., London, 2nd edition, 1907.

Plato, J., Ueber die "vitale" Färbbarkeit der Phagocyten des Menschen und einiger Säugethiere mit Neutralroth, *Arch. mikr. Anat.*, 1900, lvi, 868.

Smyth, H. F., The reactions between bacteria and animal tissues under conditions of artificial cultivation, *J. Exp. Med.*, 1915, xxi, 103.

Symmers, D., Observations on the cytology of multiple non-inflammatory necrosis of the liver and on certain related degenerative changes in cells, *J. Exp. Med.*, 1907, ix, 64.

## EXPLANATION OF PLATES.

### Plate 18.

Fig. 1. 48 hour growth of mesothelium from the intestine of an 8 day chick embryo, 2½ hours after inoculation with *B. typhosus*.

Fig. 2. 48 hour culture of the intestine of an 8 day chick embryo. Vacuolated endodermal membrane due to the presence of *B. typhosus* in the hanging drop for 2½ hours.

### Plate 19.

Fig. 3. 48 hour culture of the intestine of an 8 day chick embryo. Bacilli were moving rapidly in a vacuole in the elongated binucleate cell near the center of the field. The other organisms seen in the photograph became fastened to the growth upon fixation. They were not within the living cells but moved freely in and out of the field of vision. The cells show the vacuoles arranged around the centrosphere at one side of the nucleus as described by Lewis (1919, *a*).

Fig. 4. 48 hour growth of the connective tissue from the intestine of an 8 day chick embryo after inoculation with *B. typhosus*. 1 hour after the introduction of the organism the cells were full of small vacuoles. These later became larger and fewer in number.

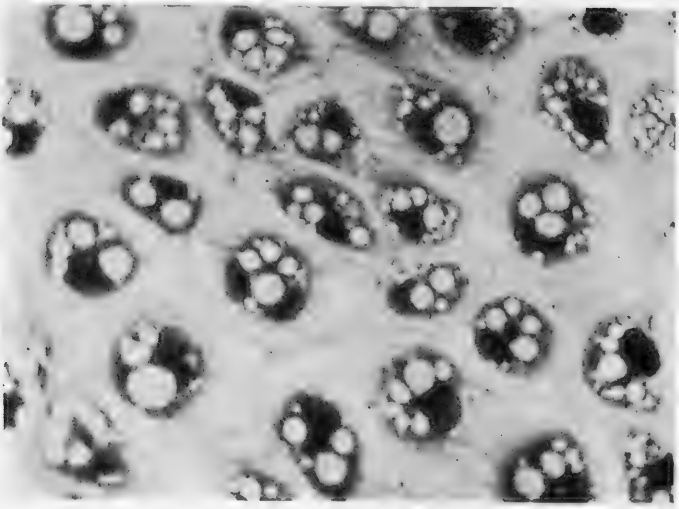

FIG. 1.

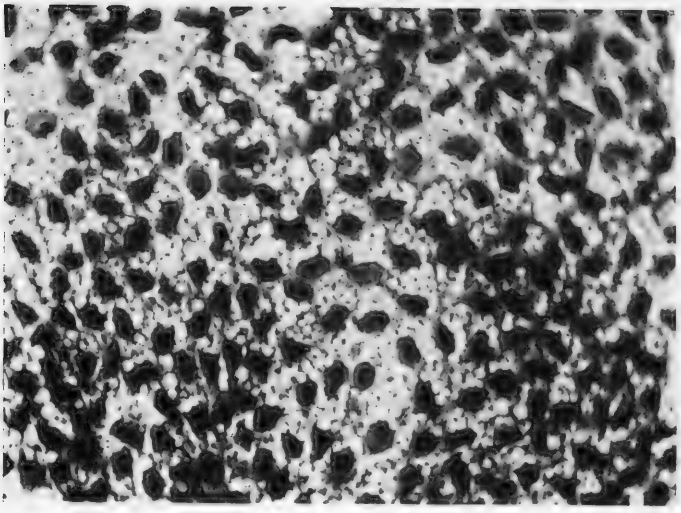

FIG. 2.

(Lewis: Vacuoles in cells of tissue cultures.)

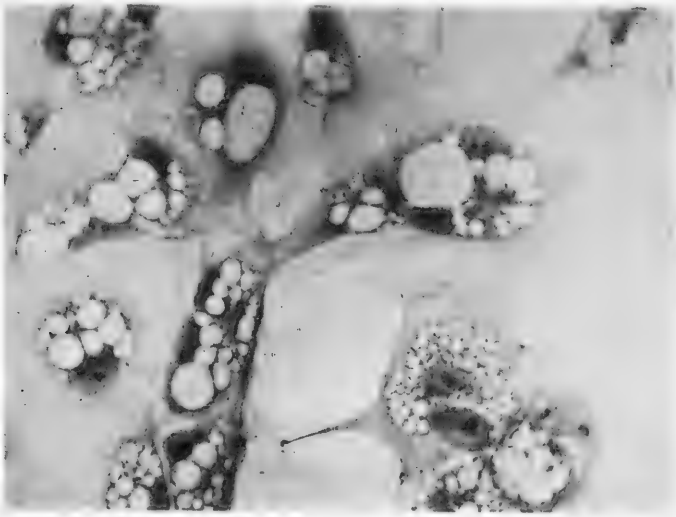

FIG. 3.

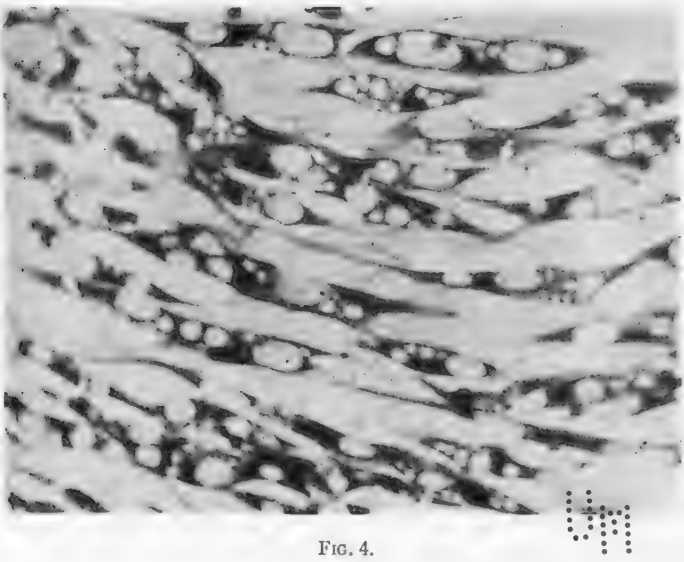

FIG. 4.

(Lewis: Vacuoles in cells of tissue cultures.)

# APPEARANCE OF ISOAGGLUTININS IN INFANTS AND CHILDREN.

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The presence of isoagglutinins in human blood, first described by Shattock and Landsteiner (1900, 1901) has attracted a great deal of attention, especially since the introduction of blood transfusion and the recognition of reactions that may follow in some instances. At the suggestion of Dr. W. L. Moss, I undertook in 1914 to determine whether isoagglutinins, as present in the blood of adults, are present at birth, and if not, at what period of life they are established.

The presence of isoagglutinins in the blood of adults regardless of health or disease has been well established, and it is not intended here to state more than the fundamental facts of adult isoagglutination before passing to the question of infants.

Landsteiner (1900, 1901) noted that when the serum of several individuals was tested against the corpuscles of the same individuals, in a certain proportion clumping of the corpuscles, or isoagglutination, occurred.

Shattock at the same time (1900) noticed that when the serum of patients with pneumonia, erysipelas, typhoid, and acute rheumatism was added to normal human blood, clumping of the corpuscles, or agglutination, occurred. This he considered a pathological phenomenon since it was not found when normal human blood serum was added to normal blood. Donath found that the serum of patients with various blood diseases (chlorosis, secondary anemia, leucemia) agglutinated normal red blood corpuscles, whereas normal human sera would not agglutinate normal human red cells. Grünbaum observed that sera from cases of typhoid fever and scarlet fever would clump the corpuscles of a patient with another disease, but would not clump corpuscles from a patient with the same disease. Eisenberg found that the serum of normal individuals seldom (1 in 10) agglutinates the corpuscles of healthy individuals, but in a large series of patients ill with various diseases agglutination of the corpuscles frequently occurred. Lo

Monaco and Panichi noticed agglutination of the corpuscles in cases of malaria. Ascoli observed this reaction in both well and sick individuals, but in the latter in greater intensity. Landsteiner (1901) showed that the isoagglutination reaction occurred with normal blood, and divided individuals into three classes, according to the reaction, as follows:

Group A. Serum agglutinates corpuscles of Group B. Corpuscles are agglutinated by sera of Groups B and C.

Group B. Serum agglutinates corpuscles of Group A. Corpuscles are agglutinated by sera of Groups A and C.

Group C. Serum agglutinates corpuscles of Groups A and B. Corpuscles are not agglutinated by sera of either Group A or B.

Later, Langer, von Decastello and Sturli, Ascoli, and Hektoen confirmed the fact that the phenomenon of isoagglutination is independent of health or disease. Moss (1910) and Janský independently showed that the classification of Landsteiner was incomplete in that if the serum of a sufficiently large group of individuals is tested against the corpuscles of each of the group, some would be found who do not fall into any of the three groups of Landsteiner (Moss' Group I). Moss classified individuals as follows:

Group I. Serum agglutinates no corpuscles. Corpuscles are agglutinated by sera of Groups II, III, and IV.

Group II. Serum agglutinates corpuscles of Groups I and III. Corpuscles are agglutinated by sera of Groups III and IV.

Group III. Serum agglutinates corpuscles of Groups I and II. Corpuscles are agglutinated by sera of Groups II and IV.

Group IV. Serum agglutinates corpuscles of Groups I, II, and III. Corpuscles are agglutinated by no sera.

These relations may also be tabulated as follows:

| Serum. | Corpuscles. | | | |
|---|---|---|---|---|
| | Group I. | Group II. | Group III. | Group IV. |
| Group I | 0 | 0 | 0 | 0 |
| " II | + | 0 | + | 0 |
| " III | + | + | 0 | 0 |
| " IV | + | + | + | 0 |

Moss proposed the following hypothesis as a probable explanation of the various isoagglutinin groups.

Group I. Serum contains no agglutinin. Corpuscles possess receptors a, b, and c.

Group II. Serum contains agglutinin A. Corpuscles possess receptors b and c.

Group III. Serum contains agglutinin B. Corpuscles possess receptors a and c.

Group IV. Serum contains agglutinin C. Corpuscles possess no receptors.

Von Dungern and Hirschfeld (1909–10, 1910, *b*, 1910–11) working at the same time found that individuals fall into four groups which they designated as follows:

Group AB, corresponding to Moss' Group I.
Group A, corresponding to Moss' Group II.
Group B, corresponding to Moss' Group III.
Group O, corresponding to Moss' Group IV.

They assumed that the agglutination reactions are due to the presence of two agglutinin radicals, A and B, in the corpuscles, and two agglutinins, $\alpha$ and $\beta$, in the serum, and explained the presence of the phenomena on the following basis.

Group AB (5.7 per cent) contains in the serum no agglutinin; contains in the corpuscles receptors A and B.

Group A (47.3 per cent) contains in the serum agglutinin $\beta$; contains in the corpuscles receptor A.

Group B (11 per cent) contains in the serum agglutinin $\alpha$; contains in the corpuscles receptor B.

Group O (36 per cent) contains in the serum agglutinins $\alpha$ and $\beta$; contains in the corpuscles no receptors.

Therefore, the serum of Group A agglutinates the corpuscles of Groups B and AB because they both contain the receptor B. The serum of Group B agglutinates the corpuscles of Groups A and AB, both containing the receptor A. The serum of Group AB agglutinates no corpuscles as the serum has no agglutinin. The serum of Group O agglutinates corpuscles of Groups A, B, and AB, as they contain receptor A or B, or both. These authors believe that the characters A and B are inherited according to the Mendelian law of heredity. Epstein and Ottenberg have also advanced this theory.

It will be seen that there are at least two, possibly more, explanations to account for the interagglutination reactions of human serum and corpuscles.

Various authors report the presence of isoagglutinins in animals (dogs, cats, steers, monkeys, etc. (Kahn and Ottenberg; Ottenberg, Friedman, and Kaliski; von Dungern and Hirschfeld (1909–10, 1910, *a*); Ingebrigtsen; Fishbein), but have failed to show that groups exist within an animal species as in the case of man.

As to the mechanism of production of isoagglutinins in the blood of man no definite proof has been forthcoming, although several explanations have been offered; *i.e.*, that isoagglutinins pass through the chorion from mother to fetus, that they are formed in infancy by an immunity reaction like bacterial agglutinins, that they are absorbed through the milk of the mother by the infant, that they are formed as the result of an immunity reaction against bacteria of the intestinal tract, and finally, that they are formed by the disintegration of red corpuscles in the individual (Halban, Ascoli, Eisenberg, Langer).

Although the isoagglutination reaction may be obtained with the blood of all adults, this is not universally true of infants. Halban was the first to study the isoagglutination reaction of maternal and fetal blood (blood from the umbilical cord at birth). He found that the agglutinating power of maternal serum was

greater than that of fetal serum as tested against adult and fetal corpuscles, and that maternal corpuscles were more readily agglutinated than were fetal corpuscles. He states that agglutinins do not necessarily pass from mother to fetus as the mother's blood may possess agglutinins which may be absent in the infant's blood.

Halban and Landsteiner found that mother's blood possessed a greater content of isoagglutinins as tested against rabbit blood than does infant's blood. Langer found that the serum of the newly-born agglutinated corpuscles of the mother in only three of eleven cases tested and seldom agglutinated adult corpuscles or corpuscles of other newly-born; he also found that the corpuscles of blood from the cord and from the newly-born were more often agglutinated by the serum of the newly-born and adults than were adult corpuscles by the serum of the newly-born, and finally, that the mother might possess isoagglutinins which were absent in the newly-born, which shows that isoagglutinins do not pass from the mother through the chorion to the fetus.

Von Decastello and Sturli tested the serum of newly-born infants (umbilical cord) against the corpuscles of the mother and of other adults, and found in a small series of seven tested that the blood of infant and mother may give different agglutination reactions. They noted further that the serum of the newly-born is usually weaker in agglutinin than that of the adult, and that agglutination is occasionally absent. In a series of eleven infants, 7 days to 4 months old, they noted frequently the absence of agglutinins in the serum, and concluded that with most young children the reaction is not so clear-cut as with adults. They state that serum soon after birth acquires agglutinin, and later the corpuscles themselves acquire agglutinophilic receptors by an immunization process.

Von Graff and von Zubrzycki found that serum of blood from the umbilical cord is poorer in agglutinin against mother's corpuscles than is the mother's serum against corpuscles of other adults. Schenk (1904) tested the interagglutination reactions between mother's and infant's blood, and concluded that the sera of mothers seldom agglutinated corpuscles of their own children, and that the serum of the child is inactive against the corpuscles of the mother. In other words, the sera of the one and corpuscles of the other behave as if they belonged to the same individual. Recently Cherry and Langrock tested the blood of thirty infants against the mothers' blood and found no agglutination, and so concluded that one can safely use mother's blood for the transfusion of her own infant. In summary, then, it has been noted that blood from the fetal cord and the blood of newly-born infants is poorer in isoagglutinin than that of adults, and that the isoagglutinin is frequently absent.

In view of the uncertainty regarding isoagglutination of infants it was undertaken to determine (1) whether the isoagglutination groups as they occur in adults are present at birth; (2) if not present at birth, how soon after birth they are established; and (3) the order in which

the serum acquires agglutinins for the four adult groups of corpuscles, and the order in which the corpuscles become agglutinable by the four adult groups of sera.

*Method.*

In order to determine whether isoagglutinin was present in the sera of the infants studied, they were tested against the corpuscles of the four adult groups, and similarly, to determine whether the corpuscles of the infants were agglutinable they were tested against sera of the four adult groups. This procedure possessed the advantage of showing whether the serum or corpuscles of the infant fell into any of the established four adult groups. Serum and corpuscles were freshly obtained from infants and adults each time the tests were made. A large series of adults was therefore grouped in order to have available at any time the fresh serum and corpuscles of the four groups.[1]

With a Wright tube a small amount of blood was drawn from the finger of the individual (adult) to be tested, sealed, allowed to clot, and centrifuged. One drop of this serum was placed on a cover-slip with a glass capillary pipette with one drop of a dilute suspension of washed red blood corpuscles of a known Group II, stirred, and inverted on a hollow ground slide. Similarly a drop of serum was placed on another cover-slip with a drop of a dilute suspension of washed red blood corpuscles of a known Group III, incubated for 1 hour, and read microscopically for agglutination. In this way, a large number of ward patients and laboratory workers were grouped. The following proportion of groups in several hundred adults tested was found.

| | *per cent* |
|---|---|
| Group I | 3 |
| Group II | 35 |
| Group III | 12 |
| Group IV | 50 |
| | 100 |

The serum and corpuscles were then obtained from each of the four adult groups—the serum in Wright tubes, which were centrifuged and the serum separated; the corpuscles collected in a tube containing

[1] In this paper the numbers of the groups are those used by Moss (1910).

1.5 per cent sodium citrate in 0.85 per cent sodium chloride and washed twice in 0.85 per cent sodium chloride. The serum and corpuscles were obtained fresh each time the tests were to be made. In a similar manner serum and corpuscles were obtained from a number of infants. To a drop of each of the four adult group corpuscles a drop of infant's serum was added. Similarly, to a drop of each of the four adult group sera a drop of infant's corpuscles was added. This may be represented diagrammatically as follows:

| | Corpuscles. | | | |
|---|---|---|---|---|
| | Group I. | Group II. | Group III. | Group IV. |
| Infant serum..... | | | | |

| | Sera. | | | |
|---|---|---|---|---|
| | Group I. | Group II. | Group III. | Group IV. |
| Infant corpuscles..... | | | | |

This makes eight tests for each infant's blood. The preparations were examined immediately to see whether the cells were evenly distributed and no false clumping was present, as may occur from insufficient washing or shaking, and were then incubated at 37.5°C. and read at the end of $\frac{1}{2}$, 1, and 2 hours.[2] The microscopic method was used throughout. It was found to be more accurate than the macroscopic for the reading of slight degrees of agglutination. By referring to the tabulation on page 314 the group of the unknown serum or corpuscles can be read.

## RESULTS.

Fetal blood, that is blood taken from the umbilical cord at birth, was obtained first, and then the blood of infants and older children. The results of the reaction as tested against adult group corpuscles and sera are shown in Tables I to V.

[2] Great care was taken to procure clean glassware. The hollow ground slides were washed in boiling water, dried, placed in 95 per cent alcohol, and dried as used. The cover-slips were boiled, put in 95 per cent alcohol, and wiped clean as used. The glass capillary pipettes and centrifuge tubes for collecting red blood corpuscles were sterilized by dry heat. The salt and citrate solutions used were sterile.

TABLE I.

*Blood from the Umbilical Cord and Infants up to 1 Month of Age.*

| No. of infant. | Race. | Sex. | Date. | Age. | Infant's serum *versus* corpuscles of. | | | | Infant's serum group. | Infant's corpuscles *versus* serum of. | | | | Infant's corpuscles group. | Infant's group serum and corpuscles. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Group I. | Group II. | Group III. | Group IV. | | Group I. | Group II. | Group III. | Group IV. | | |
| | | | *1914* | *days* | | | | | | | | | | | |
| 1 | White. | M. | June 4 | 11 | 0* | 0 | 0 | 0 | I | 0 | + | + | + | I | I |
| 2 | Negro. | F. | " 4 | 4 | 0 | 0 | 0 | 0 | | 0 | + | 0 | + | III | |
| | | | " 9 | 9 | 0 | 0 | 0 | 0 | | 0 | + | 0 | + | III | |
| 3 | " | " | " 4 | 10 | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | | |
| 4 | White. | M. | " 4 | 1 | 0 | 0 | 0 | 0 | | 0 | 0 | + | + | II | |
| | | | " 9 | 6 | 0 | 0 | 0 | 0 | | 0 | 0 | + | + | II | |
| 5 | " | F. | " 4 | 2 | 0 | 0 | 0 | 0 | | 0 | 0 | + | + | II | |
| | | | " 15 | 13 | 0 | 0 | 0 | 0 | I | 0 | + | + | + | I | I |
| 6 | " | " | " 4 | 5 | + | + | 0 | 0 | III | 0 | 0 | 0 | 0 | | |
| | | | " 6 | 7 | + | + | 0 | 0 | III | 0 | 0 | 0 | 0 | | |
| 7 | " | M. | " 4 | F.B.† | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | | |
| | | | " 6 | 2 | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | | |
| | | | " 16 | 12 | +? | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | | |
| 8 | " | F. | " 5 | F.B. | 0 | 0 | 0 | 0 | | 0 | 0 | + | + | II | |
| | | | " 18 | 13 | 0 | 0 | 0 | 0 | I | 0 | + | + | + | I | I |
| 9 | Negro. | " | " 6 | F.B. | 0 | 0 | 0 | 0 | | 0 | 0 | + | + | II | |
| | | | " 9 | 3 | 0 | 0 | 0 | 0 | | 0 | 0 | + | + | II | |
| | | | " 16 | 10 | 0 | 0 | 0 | 0 | I | 0 | + | + | + | I | I |
| 10 | White. | " | " 6 | F.B. | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | | |
| 11 | " | M. | " 6 | 7 | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | | |
| 12 | Negro. | F. | " 6 | 7 | ‡ | ‡ | ‡ | ‡ | | 0 | 0 | 0 | + | | |
| | | | " 16 | 17 | 0 | 0 | 0 | 0 | | 0 | 0 | + | + | II | |
| 13 | White. | M. | " 6 | 10 | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | +w | | |
| 14 | " | " | " 7 | F.B. | 0 | 0 | 0 | 0 | | 0 | 0 | + | + | II | |
| | | | " 16 | 9 | 0 | 0 | 0 | 0 | I | 0 | + | + | + | I | I |
| 15 | " | " | " 8 | F.B. | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | + | | |
| | | | " 18 | 10 | 0 | 0 | 0 | 0 | | 0 | + | 0 | + | III | |
| 16 | Negro. | F. | " 9 | F.B. | 0 | 0 | 0 | 0 | | ‡ | ‡ | ‡ | ‡ | | |
| | | | " 18 | 10 | 0 | 0 | 0 | 0 | | 0 | 0 | + | + | II | |
| 17 | White. | " | " 9 | 3 | 0 | 0 | 0 | 0 | | 0 | 0 | + | + | II | |
| 18 | " | " | " 9 | 2 | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | | |
| | | | " 18 | 11 | +w | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | | |

* In the tables + indicates definite agglutination; +?, doubtfully positive; +w, weakly positive; 0?, doubtful or very slight; 0, no agglutination.

† F.B. (fetal blood) is blood obtained from the umbilical cord at birth.

‡ Test not done.

TABLE I—*Concluded.*

| No. of infant. | Race. | Sex. | Date. | Age. | Infant's serum *versus* corpuscles of. | | | | Infant's serum group. | Infant's corpuscles *versus* serum of. | | | | Infant's corpuscles group. | Infant's group serum and corpuscles. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Group I. | Group II. | Group III. | Group IV. | | Group I. | Group II. | Group III. | Group IV. | | |
| | | | *1914* | *days* | | | | | | | | | | | |
| 19 | Negro. | M. | June 9 | 5 | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | | |
| | | | " 16 | 12 | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | | |
| 20 | " | F. | " 10 | F.B. | 0 | 0 | 0 | 0 | | 0 | 0 | + | + | II | |
| | | | " 18 | 9 | + | 0 | 0 | 0 | | 0 | 0 | + | + | II | |
| 21 | " | F. | " 11 | F.B. | 0 | 0 | 0 | 0 | | 0 | + | 0? | + | III? | |
| 22 | White. | " | " 13 | " | 0 | 0 | 0 | 0 | | 0 | 0 | + | + | II | |
| | | | " 18 | 5 | 0 | 0 | 0 | 0 | I | 0 | + | + | + | I | I |
| 23 | " | M. | " 16 | F.B. | 0 | 0 | 0 | 0 | I | 0 | + | + | + | I | I |
| 24 | " | F. | " 16 | " | 0 | 0 | 0 | 0 | | 0 | 0 | + | + | II | |
| 25 | Negro. | M. | " 16 | " | 0 | 0 | 0 | 0 | | 0 | 0 | + | + | II | |
| 26 | White. | F. | " 18 | " | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | | |
| 27 | " | M. | " 16 | " | 0 | 0 | 0 | 0 | | 0 | 0 | + | + | II | |
| | | | *1915* | | | | | | | | | | | | |
| 28 | " | F. | Mar. 8 | 12 | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | | |
| 29 | " | " | " 8 | 6 | 0 | 0 | 0 | 0 | | 0 | 0 | + | + | II | |
| 30 | " | M. | " 8 | 9 | 0 | 0 | 0 | 0 | | 0 | 0 | + | + | II | |
| 31 | " | F. | " 8 | 5 | 0 | 0 | 0 | 0 | | 0 | 0 | + | + | II | |
| 32 | " | " | " 12 | 4 | 0 | 0 | 0 | 0 | | 0 | + | 0 | + | III | |
| 33 | Negro. | M. | " 12 | 6 | 0 | 0 | 0 | 0 | | 0 | 0 | + | + | II | |
| 34 | White. | F. | " 12 | 3 | 0 | 0 | 0 | 0 | | 0 | 0 | + | + | II | |
| 35 | " | M. | Apr. 15 | 21 | 0 | 0 | 0 | 0 | | 0 | 0 | + | + | II | |
| 36 | " | " | " 24 | 21 | 0 | 0 | 0 | 0 | | 0 | 0? | 0 | + | III? | |
| | | | *1916* | | | | | | | | | | | | |
| 37 | " | " | Feb. 17 | 11 | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | | |
| 38 | " | " | May 25 | 8 | + | + | + | 0 | IV | 0 | 0 | 0 | 0 | IV | IV |
| 39 | " | F. | " 16 | 12 | ‡ | 0 | + | 0 | | 0 | 0 | 0 | 0 | | |
| 40 | " | " | " 16 | 10 | 0 | 0 | 0 | 0 | | 0 | 0 | +w | + | II | |
| 41 | " | " | " 16 | 11 | ‡ | 0 | 0 | 0 | | 0 | 0 | +w | + | II | |
| 42 | " | M. | " 16 | 20 | ‡ | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | | |
| 43 | " | F. | " 16 | 16 | ‡ | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | | |
| 44 | Negro. | " | " 25 | 2 | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | | |
| 45 | " | " | " 25 | 3 | + | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | | |
| 46 | White. | M. | " 25 | 12 | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | | |
| 47 | " | " | " 25 | 7 | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | + | | |
| 48 | Negro. | F. | " 25 | 5 | 0 | 0 | 0 | 0 | | 0 | + | 0 | + | III | |
| 49 | White. | M. | " 16 | 7 | 0 | 0 | 0 | 0 | | 0 | 0 | +w | + | II | |
| | | " | " 25 | 16 | 0 | 0 | 0 | 0 | | 0 | 0 | + | + | II | |

In Table I is shown the results of 67 tests on 49 infants, the blood being obtained from birth up to the age of 1 month. The striking points may be summarized as follows: (1) At birth the group is es-

TABLE II.

*Blood from Infants 1 to 3 Months of Age.*

| No. of infant. | Race. | Sex. | Date. | Age. | Infant's serum *versus* corpuscles of. | | | | Infant's serum group. | Infant's corpuscles *versus* serum of. | | | | Infant's corpuscles group. | Infant's group serum and corpuscles. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Group I. | Group II. | Group III. | Group IV. | | Group I. | Group II. | Group III. | Group IV. | | |
| | | | *1914* | *mos.* | | | | | | | | | | | |
| 50 | White. | M. | June 11 | 3 | + | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | | |
| 51 | Negro. | F. | " 11 | 1 | + | 0 | 0 | 0 | | 0 | + | 0 | + | III | |
| 52 | " | " | " 14 | 1 | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | | |
| 53 | White. | " | " 14 | 2 | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | | |
| 54 | " | " | " 18 | 1 | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | | |
| | | | *1915* | *wks.* | | | | | | | | | | | |
| 55 | Negro. | " | Feb. 17 | 7 | + | + | + | 0 | IV | 0 | 0 | 0 | 0 | IV | IV |
| | | | | *mos.* | | | | | | | | | | | |
| 56 | White. | " | " 17 | 2½ | 0 | 0 | 0 | 0 | | 0 | + | 0 | + | III | |
| 57 | " | " | " 17 | 2½ | 0 | 0 | 0 | 0 | I | 0 | + | + | + | I | I |
| | | | | *wks.* | | | | | | | | | | | |
| 58 | " | M. | " 20 | 10 | 0 | 0 | 0 | 0 | | 0 | 0 | + | + | II | |
| 59 | " | " | " 23 | 7 | 0 | 0 | 0 | 0 | | 0 | 0 | + | + | II | |
| | | | Mar. 11 | 9 | 0 | 0 | 0 | 0 | I | 0 | + | + | + | I | I |
| 60 | Negro. | " | Feb. 23 | 7 | 0 | 0 | 0 | 0 | | 0 | 0 | + | + | II | |
| | | | *1916* | *mos.* | | | | | | | | | | | |
| 61 | White. | F. | Apr. 15 | 1⅓ | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | | |
| | | | May 5 | 2 | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | | |
| 62 | " | M. | Apr. 21 | 2 | $+^w$ | $+^w$ | 0 | 0 | III | 0 | 0 | 0 | 0 | | |
| 63 | " | F. | " 24 | 2½ | $+^w$ | 0 | $+^w$ | 0 | II | 0 | 0 | + | + | II | II |
| 64 | Negro. | M. | " 25 | 2 | + | $0^?$ | + | 0 | II | 0 | 0 | 0 | 0 | | |
| 65 | White. | F. | Dec. 6 | 2 | 0 | 0 | 0 | 0 | | 0 | 0 | + | + | II | |
| | | | | *wks.* | | | | | | | | | | | |
| 35 | " | M. | May 6 | 6 | 0 | 0 | 0 | 0 | | 0 | 0 | + | + | II | |
| 36 | " | " | June 12 | 10 | 0 | 0 | 0 | 0 | | 0 | + | 0 | + | III | |

tablished in very few instances, and the serum of a newly-born infant may contain no agglutinin and his corpuscles be inagglutinable. (2) The group was established in only one of the fifteen samples of blood obtained from the cord. (3) The corpuscles become agglutinable be-

TABLE III.

*Blood from Infants 3 to 6 Months of Age.*

| No. of infant. | Race. | Sex. | Date. | Age. | Infant's serum *versus* corpuscles of. | | | | Infant's serum group. | Infant's corpuscles *versus* serum of. | | | | Infant's corpuscles group. | Infant's group serum and corpuscles. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Group I. | Group II. | Group III. | Group IV. | | Group I. | Group II. | Group III. | Group IV. | | |
| | | | *1914* | *mos.* | | | | | | | | | | | |
| 66 | White. | M. | June 11 | 4 | 0 | 0 | 0 | 0 | | 0 | 0 | + | + | II | |
| 67 | Negro. | F. | " 11 | 3½ | + | + | 0 | 0 | III | 0 | + | 0 | + | III | III |
| 68 | White. | M. | " 18 | 3½ | 0 | 0 | 0 | 0 | I | 0 | + | + | + | I | I |
| | | | *1915* | | | | | | | | | | | | |
| 69 | " | " | Jan. 27 | 5 | + | 0 | + | 0 | II | 0 | 0 | 0 | 0 | | |
| 70 | " | F. | " 27 | 4 | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | | |
| | | | Mar. 10 | 5½ | 0 | 0 | 0 | 0 | | 0 | + | 0 | + | III | |
| 71 | " | M. | Jan. 27 | 5½ | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | | |
| 72 | " | F. | " 28 | 3¼ | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | | |
| 73 | " | M. | Feb. 6 | 5 | + | + | + | 0 | IV | 0 | 0 | 0 | 0 | IV | IV |
| 74 | " | F. | " 20 | 5½ | 0 | 0 | 0 | 0 | | ‡ | ‡ | ‡ | ‡ | | |
| | | | Mar. 6 | 6 | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | | |
| 75 | " | M. | Feb. 20 | 5 | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | | |
| 76 | Negro. | F. | " 27 | 5 | + | 0 | + | 0 | II | 0 | 0 | + | + | II | II |
| 77 | White. | " | " 27 | 4 | + | 0 | + | 0 | II | 0 | 0 | + | + | II | II |
| 78 | " | M. | " 27 | 4 | 0 | 0 | 0 | 0 | | 0 | + | 0 | + | III | |
| | | | Mar. 11 | 4½ | 0 | 0 | 0 | 0 | | 0 | + | 0 | + | III | |
| 79 | " | F. | Feb. 27 | 3 | 0 | 0 | 0 | 0 | I | 0 | + | + | + | I | I |
| 80 | " | " | Mar. 3 | 5 | + | + | +? | 0 | IV? | 0 | 0 | 0 | 0 | IV? | IV? |
| | | | *1916* | | | | | | | | | | | | |
| 81 | " | M. | Apr. 21 | 3 | 0 | 0 | 0 | 0 | | 0 | 0 | + | + | II | |
| 82 | " | F. | " 24 | 3 | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | | |
| | | | June 15 | 4¾ | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | | |
| 83 | " | M. | Apr. 18 | 3¼ | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | | |
| 84 | Negro. | " | " 21 | 4½ | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | | |
| 85 | White. | " | " 15 | 3½ | 0 | 0 | 0 | 0 | | 0 | +w | 0 | + | III | |
| | | | May 6 | 4¼ | 0 | 0 | 0 | 0 | | 0 | + | 0 | + | III | |
| | | | " 26 | 5 | 0 | 0 | 0 | 0 | | 0 | + | 0 | + | III | |
| | | | June 12 | 5½ | 0 | 0 | 0 | 0 | | 0 | + | 0 | + | III | |
| 86 | Negro. | F. | Apr. 24 | 4 | 0 | 0? | 0 | 0 | | 0 | 0 | 0 | 0 | | |
| | | | May 6 | 4½ | 0? | + | 0? | 0 | | 0 | 0 | 0 | 0 | | |
| 62 | White. | M. | " 24 | 3 | +w | +w | 0 | 0 | III | 0 | 0 | 0 | 0 | | |
| | | | June 12 | 3¾ | + | + | 0 | 0 | III | 0 | 0 | 0 | 0 | | |

‡ Test not done.

fore agglutinin is present in the serum. (4) In only seven of the 49 infants tested was agglutinin present in the serum, though the corpuscles were agglutinated in thirty-two cases. (5) Isoagglutination in

TABLE IV.

*Blood from Infants 6 to 12 Months of Age.*

| No. of infant. | Race. | Sex. | Date. | Age. | Infant's serum *versus* corpuscles of. | | | | Infant's serum group. | Infant's corpuscles *versus* serum of. | | | | Infant's corpuscles group. | Infant's group serum and corpuscles. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Group I. | Group II. | Group III. | Group IV. | | Group I. | Group II. | Group III. | Group IV. | | |
| | | | *1914* | *mos.* | | | | | | | | | | | |
| 87 | White. | M. | June 16 | 11 | + | + | 0 | 0 | III | 0 | 0 | 0 | 0 | | |
| 88 | " | F. | " 16 | 8 | + | + | 0 | 0 | III | 0 | + | 0 | + | III | III |
| 89 | " | M. | " 16 | 9 | + | + | 0 | 0 | III | 0 | 0 | 0 | 0 | | |
| 90 | Negro. | F. | " 16 | 10 | 0? | 0 | 0 | 0 | I | 0 | + | + | + | I | I |
| 91 | " | M. | " 16 | 10 | + | + | 0 | 0 | III | 0 | + | 0 | + | III | III |
| 92 | White. | " | " 14 | 7 | 0 | 0 | 0 | 0 | I | 0 | + | + | + | I | I |
| 93 | " | " | " 18 | 8 | 0 | 0 | 0 | 0 | I | 0 | + | + | + | I | I |
| | | | *1915* | | | | | | | | | | | | |
| 94 | " | " | Jan. 27 | 10 | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | | |
| 95 | " | F. | " 27 | 6 | + | + | + | 0 | IV | 0 | 0 | 0 | 0 | IV | IV |
| 71 | " | M. | Feb. 27 | 6½ | 0 | 0 | 0 | 0 | | 0 | 0 | + | + | II | |
| 96 | " | " | Jan. 28 | 6½ | + | 0 | + | 0 | II | 0 | 0 | + | + | II | II |
| | | | Mar. 6 | 7¾ | + | 0 | + | 0 | II | 0 | 0 | + | + | II | II |
| 97 | " | " | Jan. 28 | 6 | 0 | 0 | 0 | 0 | | 0 | 0 | + | + | II | |
| 98 | " | " | Mar. 5 | 10 | + | + | 0 | 0 | III | 0 | + | 0 | + | III | III |
| 99 | Negro. | " | " 5 | 10½ | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | | |
| | | | *1916* | | | | | | | | | | | | |
| 100 | White. | F. | Apr. 15 | 6½ | +w | 0 | + | 0 | II | 0 | 0 | +w | +w | II | II |
| | | | May 6 | 7¼ | + | 0 | + | 0 | II | 0 | 0 | +w | + | II | II |
| 101 | " | " | Apr. 19 | 10 | 0 | 0 | 0 | 0 | | 0 | 0 | + | + | II | |
| 102 | " | M. | " 19 | 11 | + | + | + | 0 | IV | 0 | 0 | 0 | 0 | IV | IV |
| 103 | " | " | " 19 | 8½ | + | + | + | 0 | IV | 0 | 0 | 0 | 0 | IV | IV |
| 104 | " | F. | " 21 | 9 | + | + | + | 0 | IV | 0 | 0 | 0 | 0 | IV | IV |
| 105 | " | " | " 21 | 8 | + | 0 | +w | 0 | II | 0 | 0 | + | + | II | II |
| 106 | " | " | May 6 | 7¼ | 0? | 0 | +w | 0 | | 0 | 0 | + | + | II | |
| | | | " 26 | 8 | +w | 0 | + | 0 | II | 0 | 0 | + | + | II | II |
| 36 | " | M. | Sept. 12 | 6 | + | + | 0 | 0 | III | 0 | + | 0 | + | III | III |
| 82 | " | F. | " 12 | 7¾ | + | + | 0 | 0 | III | 0 | + | 0 | + | III | III |

such a way as to allow classification of the infant in one of the fixed adult groups occurred in only eight of the 49 cases tested. Of these one belonged to Group IV, and the other seven to Group I. No mem-

TABLE V.

*Blood from Children 1 to 10½ Years of Age.*

| No. of child. | Race. | Sex. | Date. | Age. | Child's serum *versus* corpuscles of. | | | | Child's serum group. | Child's corpuscles *versus* serum of. | | | | Child's corpuscles group. | Child's group serum and corpuscles. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Group I. | Group II. | Group III. | Group IV. | | Group I. | Group II. | Group III. | Group IV. | | |
| | | | *1914* | | | | | | | | | | | | |
| 107 | White. | M. | June 11 | 13 mos. | + | + | 0 | 0 | III | 0 | + | 0 | + | III | III |
| | | | *1915* | | | | | | | | | | | | |
| | | | Feb. 2 | 21 " | + | + | 0 | 0 | III | 0 | + | 0 | + | III | III |
| | | | *1914* | | | | | | | | | | | | |
| 108 | Negro. | F. | June 14 | 18 " | + | + | + | +* | IV? | 0 | 0 | 0 | 0 | IV? | IV? |
| 109 | " | M. | " 18 | 2 yrs.,1 mo. | + | + | 0 | 0 | III | 0 | 0 | 0 | 0 | | |
| | | | *1915* | | | | | | | | | | | | |
| 110 | White. | " | Jan. 27 | 3 " 7 mos. | + | 0 | + | 0 | II | 0 | 0 | + | + | II | II |
| 111 | " | " | " 27 | 4 " 1 mo. | + | 0 | + | 0 | II | 0 | 0 | + | + | II | II |
| 112 | " | " | " 27 | 5 " 2 mos. | 0 | 0 | 0 | 0 | I | 0 | + | + | + | I | I |
| 113 | Negro. | F. | " 27 | 3 " 2 " | + | + | 0 | 0 | III | 0 | + | 0 | + | III | III |
| | | | Mar. 5 | 3 " 3 " | + | + | 0 | 0 | III | 0 | + | 0 | + | III | III |
| 114 | " | M. | Jan. 28 | 19 mos. | + | + | + | 0 | IV | 0 | 0 | 0 | 0 | IV | IV |
| 115 | White. | " | Feb. 2 | 20 " | + | + | + | 0 | IV | 0 | 0 | 0 | 0 | IV | IV |
| 116 | " | F. | " 2 | 10½ yrs. | + | + | 0 | 0 | III | 0 | + | 0 | + | III | III |
| 117 | " | " | " 2 | 5½ " | + | + | + | 0 | IV | 0 | 0 | 0 | 0 | IV | IV |
| 118 | " | " | " 2 | 4½ " | + | 0 | + | 0 | II | 0 | 0 | + | + | II | II |
| 119 | " | " | " 4 | 8½ " | + | + | + | 0 | IV | 0 | 0 | 0 | 0 | IV | IV |
| 120 | " | " | " 4 | 3 yrs., 8 mos. | + | + | 0 | 0 | III | 0 | + | 0 | + | III | III |
| 121 | " | M. | " 4 | 3 yrs. | + | + | + | 0 | IV | 0 | 0 | 0 | 0 | IV | IV |
| 122 | " | " | " 5 | 2 yrs., 9 mos. | + | 0 | + | 0 | II | 0 | 0 | + | + | II | II |
| 123 | " | " | " 6 | 3 " 9 " | + | 0 | + | 0 | II | 0 | 0 | + | + | II | II |
| 124 | Negro. | " | " 6 | 2 yrs. | + | + | + | 0 | IV | 0 | 0 | 0 | 0 | IV | IV |
| 125 | White. | " | " 20 | 2 yrs., 5 mos. | + | + | 0 | 0 | III | 0 | + | 0 | + | III | III |
| 126 | " | " | Mar. 3 | 17 mos. | + | + | + | 0 | IV | 0 | 0 | 0 | 0 | IV | IV |
| | | | *1914* | | | | | | | | | | | | |
| 127 | " | " | June 18 | 16½ " | 0 | 0 | 0 | 0 | I | 0 | + | + | + | I | I |
| | | | *1916* | | | | | | | | | | | | |
| 128 | " | " | Apr. 24 | 12 " | + | + | + | 0 | IV | 0 | 0 | 0 | 0 | IV | IV |
| 129 | " | F. | " 24 | 22 " | + | + | + | 0 | IV | 0 | 0 | 0 | 0 | IV | IV |
| 130 | " | " | " 15 | 2 yrs. | + | 0 | + | 0 | II | 0 | 0 | + | + | II | II |
| 131 | Negro. | " | " 21 | 2 " | + | 0 | + | 0 | II | 0 | 0 | + | + | II | II |
| 70 | White. | " | May 2 | 19 mos. | + | + | 0 | 0 | III | 0 | + | 0 | + | III | III |
| 80 | " | " | " 2 | 19 " | + | + | + | 0 | IV | 0 | 0 | 0 | 0 | IV | IV |
| 68 | " | M. | " 2 | 2 yrs., 2 mos. | 0 | 0 | 0 | 0 | I | 0 | + | + | + | I | I |
| 87 | " | " | " 2 | 2 " 10 " | + | + | + | 0 | IV | 0 | 0 | 0 | 0 | IV | IV |
| 92 | " | " | " 2 | 2 " 5 " | 0 | 0 | 0 | 0 | I | 0 | + | + | + | I | I |
| 58 | " | " | " 2 | 17 mos. | + | 0 | + | 0 | II | 0 | 0 | + | + | II | II |
| 75 | " | " | " 2 | 19½ " | + | + | + | 0 | IV | 0 | 0 | 0 | 0 | IV | IV |

* Serum agglutinated corpuscles of all four adult groups. Serum heated to 56°C. for 30 minutes still agglutinated all four groups of corpuscles.

bers of Group II or III were present. (6) The corpuscles may be agglutinated by a serum of Group IV only, or by the serum of Groups II and IV, or III and IV, without the serum possessing any agglutinins, and an infant's corpuscles that are agglutinated by the serum of

TABLE VI.

*Summary of Agglutination Reactions.*

| Table No. | No. of individuals tested. | Age. | Corpuscles agglutinated. | | Serum contained agglutinin. | | Serum contained agglutinin; corpuscles agglutinated; *i.e.*, fixed group. | | Cases in which group was established. |
|---|---|---|---|---|---|---|---|---|---|
| | | | No. of infants. | Per cent. | No. of infants. | Per cent. | No. of infants. | Per cent. | |
| I | 49 | Birth to 1 mo. | 32 | 65.3 | 7 | 14.2 | 8 | 16.3 | Group I, 7<br>" II, 0<br>" III, 0<br>" IV, 1 |
| II | 18 | 1–3 mos. | 10 | 55.5 | 6 | 33.3 | 4 | 22.2 | " I, 2<br>" II, 1<br>" III, 0<br>" IV, 1 |
| III | 22 | 3–6 " | 10 | 45.4 | 8 | 36.3 | 7 | 31.8 | " I, 2<br>" II, 2<br>" III, 1<br>" IV, 2 |
| IV | 23 | 6–12 " | 15 | 65.2 | 15 | 65.2 | 16 | 69.5 | " I, 3<br>" II, 4<br>" III, 5<br>" IV, 4 |
| V | 32 | 1–10½yrs. | 18 | 56.2 | 28 | 87.5 | 31 | 96.8 | " I, 4<br>" II, 8<br>" III, 6<br>" IV, 13 |
| Total... | 144 | | | | | | | | |

Groups III and IV may later acquire receptors for the serum of Group II, the infant thus becoming a member of Group I. (7) The serum may possess agglutinin against the corpuscles of Group I only, or Groups I and II, or Groups I and III, or in one case Groups I, II, and III (Group IV).

In Table II are seen the results of twenty tests on eighteen infants, 1 to 3 months old. In these, agglutination of the corpuscles took place in ten cases and agglutinin was found in the serum in six. The group was established in serum and corpuscles in only four of the eighteen cases.

In Table III thirty-one tests on twenty-two infants, 3 to 6 months of age, showed that agglutination of the corpuscles occurred in ten cases, and agglutinins in the serum in eight cases; the group was established in serum and corpuscles in seven of the twenty-two.

In Table IV twenty-six tests on twenty-three infants, 6 to 12 months of age, showed that agglutination of the corpuscles occurred in fifteen cases, and agglutinins were present in the serum in fifteen; the group was established in serum and corpuscles in sixteen cases.

Table V shows the results of thirty-four tests on thirty-two children, 1 to $10\frac{1}{2}$ years of age. Agglutination of the corpuscles took place in eighteen cases, and agglutinins were present in the serum in twenty-eight; the group was established in serum and corpuscles in thirty-one of the thirty-two.

These results are summarized in Table VI.

*Results of Successive Agglutination Tests on the Same Infant.*

By referring to Tables I to V it will be noted that in thirty-eight cases the agglutination tests were repeated in the same infants' blood. In twenty-three of these there was no change in the grouping obtained. In fifteen the reaction had changed (Table VII).

These results may be summarized as follows:

Instances in which corpuscles showed no agglutination at first examination; later acquired receptors, becoming Group II..... 1
Instances in which corpuscles showed no agglutination at first examination; later acquired receptors, becoming Group III..... 4
Instances in which corpuscles showed receptors for Group II at first examination; later acquired receptors, becoming Group I..... 6
Instances in which serum showed no agglutinin at first examination; later acquired agglutinin, becoming Group II..... 2
Instances in which serum showed no agglutinin at first examination; later acquired agglutinin, becoming Group III..... 3
Instances in which serum showed no agglutinin at first examination; later acquired agglutinin, becoming Group IV..... 1
Instances in which serum showed agglutinin for Group III at first examination; later acquired agglutinin, becoming Group IV..... 1

TABLE VII.

*Changes in Agglutination Reaction on Repeated Examination.*

| No. of infant. | Age. | Infant's serum group. | Infant's corpuscles group. | Infant's blood group. |
|---|---|---|---|---|
| 5 | 2 days. | Not established. | II | Not established. |
| | 13 " | I | I | I |
| 8 | F. B. | Not established. | II | Not established. |
| | 13 days. | I | I | I |
| 9 | F. B. | Not established. | II | Not established. |
| | 4 days. | " " | II | " " |
| | 11 " | I | I | I |
| 12 | 7 days. | Not established. | Not established. | Not established. |
| | 17 " | " " | II | " " |
| 14 | F. B. | " " | II | " " |
| | 10 days. | I | I | I |
| 15 | F. B. | Not established. | Not established. | Not established. |
| | 10 days. | " " | III | " " |
| 22 | F. B. | " " | II | " " |
| | 5 days. | I | I | I |
| 36 | 3 wks. | Not established. | Not established. | Not established. |
| | 10 " | " " | III | " " |
| | 6 mos. | III | III | III |
| 59 | 7 wks. | Not established. | II | Not established. |
| | 9 " | I | I | I |
| 70 | 4 mos. | Not established. | Not established. | Not established. |
| | 5¼ " | " " | III | " " |
| | 19 " | III | III | III |
| 82 | 3 " | Not established. | Not established. | Not established. |
| | 4¾ " | " " | " " | " " |
| | 7¾ " | III | III | III |
| 75 | 5 " | Not established. | Not established. | Not established. |
| | 19 " | IV | IV | IV |

TABLE VII—*Concluded.*

| No. of infant. | Age. | Infant's serum group. | Infant's corpuscles group. | Infant's blood group. |
|---|---|---|---|---|
| 87 | 11 mos.<br>2 yrs.<br>10 mos. | III<br>IV | Not established.<br>IV | Not established.<br>IV |
| 58 | 10 wks.<br>17 mos. | Not established.<br>II | II<br>II | Not established.<br>II |
| 71 | 5½ "<br>6½ " | Not established.<br>" " | Not established.<br>II | Not established.<br>" " |

It is thus evident that corpuscles possessing no agglutinophilic receptors, *i.e.* not agglutinated by any sera, may acquire receptors, becoming members of Group II, III, or I, and that Group II corpuscles may change to Group I by further acquisition of receptors. This is also probably true for Group III corpuscles.

Similarly a serum containing no agglutinin, *i.e.* agglutinating no corpuscles, may acquire agglutinin for Group II, or for Group III, or for Group IV, and a serum containing agglutinin for Group III may change to Group IV by further acquisition of agglutinin. This is also probably true for Group II.

I have not seen a serum give up agglutinin which it has once acquired or corpuscles give up receptors, and do not believe that this occurs.

It is apparent, therefore, that an infant may be born with agglutinin in his blood, but it is rarely present in serum and corpuscles at birth so as to place him in a fixed group. He acquires further agglutinin first in his corpuscles and then in his serum and establishes his quota of group agglutinins usually by the end of the 1st year. After 1 year thirty-one of the thirty-two children had their group established, the one exception (No. 109, Table V) showing a Group III serum, with his corpuscles still inagglutinable. After 1 year the relative proportion of the fixed groups is approximately the same as with adults.

*Relation of the Mother's Blood Grouping to That of the Infant.*

In twenty-three cases the group of the mother was determined simultaneously with that of the infant (tested against the same group corpuscles and sera). The results are given in Table VIII.

In twenty-three cases the mothers' groups were as follows:

| | |
|---|---|
| Group I | 1 |
| Group II | 9 |
| Group III | 4 |
| Group IV | 9 |
| Total | 23 |

The infants' groups were as follows:

| | |
|---|---|
| Group not established | 16 |
| Group I | 3 |
| Group II | 1 |
| Group III | 3 |
| Group IV | 0 |
| Total | 23 |

In cases in which the infant's group was established the following results were obtained.

| | |
|---|---|
| Instances in which blood of mother and infant were of same group | 1 |
| Instances in which blood of mother and infant were of different groups | 6 |
| Instances in which mother's serum agglutinated the infant's corpuscles | 11 |
| Instances in which mother's corpuscles were agglutinated by the infant's serum | 3 |

It has been suggested by Cherry and Langrock and others that it is safe to transfuse infants with their own mother's blood without preliminary tests. It would appear from the results given above that this is not a safe practice for the possibility of changes from agglutination is frequently present. For example, the mother may belong to Group II and the infant to Group III, or *vice versa*, or the mother to Group II and the infant to Group I. This may occur in an infant as early as the 4th day (No. 2, Table VIII). It would seem safer, therefore, to make preliminary tests on all infants.

TABLE VIII.

*Relation of the Grouping in the Mother's Blood to That in the Infant's Blood.*

| No. of infant. | Mother's blood group. | Infant's age. | Infant's serum group. | Infant's corpuscles group. | Infant's blood group. |
|---|---|---|---|---|---|
| | | *days* | | | |
| 2 | II* | 4 | Not established. | III | Not established. |
| | | 9 | " " | III | " " |
| 3 | IV | 10 | " " | Not established. | " " |
| 4 | IV* | 1 | " " | II | " " |
| | | 6 | " " | II | " " |
| 5 | II* | 2 | " " | II | " " |
| | | 13 | I | I | I |
| 6 | IV | 5 | III | Not established. | Not established. |
| | | 7 | III | " " | " " |
| 7 | IV | F.B. | Not established. | " " | " " |
| | | 2 | " " | " " | " " |
| | | 12 | " " | " " | " " |
| 8 | II* | F.B. | " " | II | " " |
| | | 13 | I | I | I |
| 11 | IV | 7 | Not established. | Not established. | Not established. |
| 13 | IV | 10 | " " | " " | " " |
| | | *mos.* | | | |
| 70 | III | 4 | " " | " " | " " |
| | | $5\frac{1}{2}$ | " " | III | " " |
| | | 19 | III | III | III |
| 96 | III*† | $7\frac{3}{4}$ | II | II | II |
| 97 | II | 6 | Not established. | II | Not established. |
| | | *wks.* | | | |
| 59 | II* | 7 | " " | II | " " |
| | | 9 | I | I | I |
| | | *days* | | | |
| 28 | IV | 12 | Not established. | Not established. | Not established. |
| 29 | III* | 6 | " " | II | " " |
| 30 | II | 9 | " " | II | " " |
| 31 | II | 5 | " " | II | " " |
| 32 | III | 4 | " " | III | " " |
| 33 | IV* | 6 | " " | II | " " |
| 34 | IV* | 3 | " " | II | " " |
| | | *wks.* | | | |
| 36 | II*† | 3 | " " | Not established. | " " |
| | | 10 | " " | III | " " |
| | | *mos.* | | | |
| | | 6 | III | III | III |
| 65 | I | 2 | Not established. | II | Not established. |
| 82 | II*† | 3 | " " | Not established. | " " |
| | | $4\frac{1}{2}$ | " " | " " | " " |
| | | $7\frac{3}{4}$ | III | III | III |

* Infant's corpuscles agglutinated by mother's serum.

† Mother's corpuscles agglutinated by infant's serum.

## *Presence of Group Isoagglutinins in the Mother's Milk and Their Relation to the Infant's Blood Grouping.*

As is well known, various bacteriological antibodies have been found to pass over from the mother's blood to mother's milk, and through the milk to her nursing infant, and subsequently to appear in the infant's blood. Ehrlich first demonstrated this by immunizing female mice to abrin, ricin, swine plague, diphtheria, and tetanus toxin, and demonstrating the presence of antitoxin to these substances in the milk of the mother and through the milk to the blood of the nursing young. Brieger and Ehrlich showed that this was also true of diphtheria antitoxin. Römer (1901) found that colts nursing mares immunized to diphtheria acquire considerable diphtheria antitoxin in the blood, and Römer and Sames, and Römer and Much showed that newly-born sheep acquire immunity to tetanus by nursing immune mothers or by having the milk of immune mothers fed *per os*, and the presence of tetanus antitoxin may be demonstrated in the blood. They explained this by the fact that the intestines of the newly-born animal are permeable to foreign protein (antitoxin) which passes through to the blood. After the 3rd day little is absorbed through the intestines. They found this to be true also in a calf during the first 6 days of life. They state that more antitoxin is absorbed through the colostrum than through the milk, as colostrum contains more albumin and globulin. A number of other authors have confirmed this.

Other substances, such as alexin (Moro) and hemolysins (Pfaundler and Moro), have been shown to appear in milk.

It was first demonstrated by Widal and Siccard (1897, *a*, *b*), later by Landouzy and Griffon, Castaigne, Römer (1909), and Rosenau and McCoy that typhoid agglutinins may pass from mother to nursing infant by way of the milk. In regard to hemàgglutinins, Kraus by injecting rabbits with dog corpuscles found immune hemagglutinins in the blood and milk of rabbits, but though present in the milk they were not found in the blood of the nursing young rabbits. Langer first demonstrated isoagglutinins in the milk and colostrum of human mothers, but was unable to demonstrate them in the blood of ten nursing infants, 2 to 9 months of age, although present in mothers' milk. Schenk (1905) found that the milk of normal women contains isoagglutinin as does the colostrum. Von Zubrzycki and Wolfsgruber found normal isoagglutinins in human milk. These are present in greater proportion in the first 10 days of the puerperium than later, but they found the agglutinins present in small amounts or absent in the blood of nursing infants.

I was able to confirm this by examining the breast milk of fourteen nursing women, finding in each instance isoagglutinins present (Table IX). Furthermore, the isoagglutinins fell into groups identical with those of the mothers' blood. The groups were determined by testing the milk against corpuscles of Groups II and III, the method employed being similar to that used in testing the group of a serum.

In each instance the agglutination of the serum and corpuscles of the nursing infants was also tested. It will be seen that of the fourteen infants tested, in only five was the group established in the serum and corpuscles, although one (No. 97) had been nursing the mother for

TABLE IX.

*Relation of the Grouping in the Mother's Milk to That in the Infant's Blood.*

| No. of infant. | Date. | Mother's blood group. | Mother's milk group. | Infant's age. | Infant's serum group. | Infant's corpuscles group. | Infant's blood group. |
|---|---|---|---|---|---|---|---|
| | *1915* | | | *mos.* | | | |
| 96 | Mar. 6 | III | III | 7¾ | II | II | II |
| 97 | " 6 | II | II | 7¼ | Not established. | II | Not established. |
| | | | | *days* | | | |
| 31 | " 8 | II | III | 5 | " " | II | " " |
| 29 | " 8 | III | II | 6 | " " | II | " " |
| 30 | " 8 | II | II | 9 | " " | II | " " |
| 28 | " 8 | IV | IV | 12 | " " | Not established. | " " |
| | | | | *mos.* | | | |
| 70 | " 10 | III | III | 5½ | " " | III | " " |
| | *1916* | | | | | | |
| | May 2 | | | 19 | III | III | III |
| | *1915* | | | *wks.* | | | |
| 59 | Mar. 11 | II | II | 9 | I | I | I |
| | | | | *days* | | | |
| 33 | " 12 | IV | IV | 6 | Not established. | II | Not established. |
| 34 | " 12 | IV | IV | 3 | " " | II | " " |
| 32 | " 12 | III | III | 4 | " " | III | " " |
| | *1916* | | | *wks.* | | | |
| 36 | June 12 | ‡ | ‡ | 10 | " " | III | " " |
| | | | | *mos.* | | | |
| | Sept. 12 | II | II | 6 | III | III | III |
| 82 | Apr. 24 | ‡ | ‡ | 3 | Not established. | Not established. | Not established. |
| | Sept. 12 | II | II | 7¾ | III | III | III |
| 65 | Dec. 6 | I | I | 2 | Not established. | II | Not established. |

‡ Test not done.

7¼ months. The blood of the fourteen nursing infants had no more isoagglutinin than did the blood of other infants, artificially fed, tested at the same age, so it would appear that the infant does not acquire his hemisoagglutinin through his mother's milk.

## SUMMARY AND CONCLUSIONS.

1. The isoagglutination reaction of 131 infants and children from birth to $10\frac{1}{2}$ years was examined by testing their serum and washed corpuscles microscopically against the serum and corpuscles of each of the four adult groups.

2. The grouping as present in adults is rarely present in blood from the umbilical cord.

3. At birth and during the 1st month of life isoagglutination is rarely present, but the percentage of infants in whom the isoagglutinin group is established increases with age, so that after 1 year the group is usually established, and after 2 years is always present as in adults.

4. The grouping is established in the corpuscles before it is established in the serum; *i.e.*, the corpuscles acquire agglutinophilic receptors before the serum acquires agglutinin. Therefore, Group I is the first group to be formed and Group IV is the last.

5. The early grouping in the corpuscles before the group is established in the serum is liable to change by the acquisition of new receptors.

6. When the grouping has been established in both serum and corpuscles it does not change.

7. Isoagglutinins are present in mother's milk and the grouping is identical with that in the mother's blood. These agglutinins are probably not transmitted to the nursing infant through the milk.

8. On account of the differences between the agglutination reactions in the blood of mother and child it is not safe to transfuse an infant from its mother without making the preliminary tests.

## BIBLIOGRAPHY.

Ascoli, M., *Münch. med. Woch.*, 1901, xlviii, 1239.
Brieger and Ehrlich, *Z. Hyg. u. Infectionskrankh.*, 1893, xiii, 336.
Castaigne, J., *Compt. rend. Soc. biol.*, 1897, iv, series 10, 984.
Cherry, T. H., and Langrock, E. G., *J. Am. Med. Assn.*, 1916, lxvi, 626.
von Decastello, A., and Sturli, A., *Münch. med. Woch.*, 1902, xlix, 1090.
Donath, J., *Wien. klin. Woch.*, 1900, xiii, 497.
von Dungern, E., and Hirschfeld, L., *Z. Immunitätsforsch., Orig.*, 1909–10, iv, 531.
von Dungern, E., and Hirschfeld, L., *Münch. med. Woch.*, 1910, *a*, lvii, 741.

von Dungern, E., and Hirschfeld, L., *Z. Immunitätsforsch., Orig.*, 1910, *b*, vi, 284.
von Dungern, E., and Hirschfeld, L., *Z. Immunitätsforsch., Orig.*, 1910–11, viii, 526.
Ehrlich, P., *Z. Hyg. u. Infectionskrankh.*, 1892, xii, 183.
Eisenberg, P., *Wien. klin. Woch.*, 1901, xiv, 1020.
Epstein, A. A., and Ottenberg, R., *Tr. New York Path. Soc.*, 1908, viii, 117.
Fishbein, M., *J. Infect. Dis.*, 1913, xii, 133.
von Graff, E., and von Zubrzycki, J., *Arch. Gynäk.*, 1911–12, xcv, 732.
Grünbaum, *Brit. Med. J.*, 1900, i, 1089.
Halban, J., *Wien. klin. Woch.*, 1900, xiii, 545.
Halban, J., and Landsteiner, K., *Münch. med. Woch.*, 1902, xlix, 473.
Hektoen, L., *J. Infect. Dis.*, 1907, iv, 296.
Ingebrigtsen, R., *Münch. med. Woch.*, 1912, lix, 1475.
Janský, J., *Sborn. klin.*, 1906–07, viii, 85, quoted by Moss (1910).
Kahn, M. H., and Ottenberg, R., *J. Exp. Med.*, 1911, xiii, 536.
Kraus, R., *Wien. klin. Woch.*, 1901, xiv, 737.
Landouzy and Griffon, *Compt. rend. Soc. biol.*, 1897, iv, series 10, 950.
Landsteiner, K., *Centr. Bakteriol., 1te Abt.*, 1900, xxvii, 357.
Landsteiner, K., *Wien. klin. Woch.*, 1901, xiv, 1132.
Landsteiner, K., and Leiner, K., *Centr. Bakteriol., 1te Abt., Orig.*, 1905, xxxviii, 548.
Landsteiner, K., and Reich, M., *Centr. Bakteriol., 1te Abt., Orig.*, 1905, xxxix, 712.
Langer, J., *Z. Heilk., Abt. int. Med.*, 1903, xxiv, 111.
Lo Monaco, D., and Panichi, L., *Riforma med.*, 1902, xviii, 3, abstracted in *Münch. med. Woch.*, 1902, xlix, 1066.
Moro, E., *Jahrb. Kinderheilk.*, 1902, lv, 396.
Moss, W. L., *Bull. Johns Hopkins Hosp.*, 1910, xxi, 63.
Moss, W. L., *Bull. Johns Hopkins Hosp.*, 1911, xxii, 238.
Ottenberg, R., Friedman, S. S., and Kaliski, D. J., *Tr. New York Path. Soc.*, 1911, xi, 49.
Pfaundler, M., and Moro, E., *Z. exp. Path. u. Therap.*, 1907, iv, 451.
Römer, *Berl. klin. Woch.*, 1901, xxxviii, 1150.
Römer, H., in Sommerfeld, P., Handbuch der Milchkunde, Wiesbaden, 1909, 472.
Römer, P. H., and Much, H., *Jahrb. Kinderheilk.*, 1906, lxiii, 684.
Römer, P. H., and Sames, T., *Z. Immunitätsforsch., Orig.*, 1909, iii, 49.
Rosenau, M. J., and McCoy, G. W., *Bull. Hyg. Lab., U. S. P. H., No. 56*, 1909, 470.
Schenk, F., *Monatschr. Geburtsh. u. Gynäk.*, 1904, xix, 158, 344, 568.
Schenk, F., *Münch. med. Woch.*, 1905, lii, 1623.
Shattock, S. G., *J. Path. and Bacteriol.*, 1900, vi, 303.
Widal and Sicard, *Compt. rend. Soc. biol.*, 1897, *a*, iv, series 10, 804.
Widal and Sicard, *La Semaine méd.*, 1897, *b*, xvii, 282.
von Zubrzycki, J., and Wolfsgruber, R., *Deutsch. med. Woch.*, 1913, xxxix, 210.

# EFFECT OF FEEDING THE PINEAL BODY UPON THE DEVELOPMENT OF THE ALBINO RAT.

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Plate 20.

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In a study during the past 2 years of the relation of certain endocrine glands to growth, two series of experiments were carried out with the purpose of ascertaining more accurately the effects of feeding with the pituitary gland and the thymus.[1] The following experiments were undertaken in the hope of adding further knowledge concerning the function of the pineal body, especially in its relation to growth and early sexual changes.

## *Method.*

The method employed in our experiments consists in the oral administration of desiccated pineal powder.[2] The pineal body of young calves was minced, dried *in vacuo* at a temperature of 35–40°C., and extracted with ether. 1 gm. of this fat-free desiccated pineal substance represents 7 gm. of the fresh pineal body. The powder was given once or twice daily in doses varying from 0.01 to 0.1 gm. It was mixed with a small amount of bread and milk and the mixture was given to the rats before they received their daily rations. The animals were placed in small compartments when fed the powder, in order to insure its complete ingestion.

The albino rat (*Mus norvegicus albinus*) of the standard stock of the Wistar Colony, Philadelphia, was selected as the animal most suitable for our experimental work. This rat undergoes striking developmental changes in a relatively short period. Moreover, exten-

[1] Work not yet published.

[2] This powder was obtained from Armour and Company, Chicago.

sive studies of its normal development have been made by Donaldson,[3] whose statistics we have used for comparative study.

The experiments were begun when the rats were about 3 weeks of age, immediately after weaning. The young animals were placed in pairs, in separate compartments of specially constructed cages. Precautions were taken to secure ideal hygienic surroundings, to give protection against light and noise, and to afford ample room for the normal activities of the animals. The diet of the rats consisted of wheat, corn, bread, and milk. In the first two experiments the animals were given an excess of bread and milk and mixed grains twice daily. In the third experiment each animal was given equal weighed amounts of Graham flour and milk. The rats were fed liberally and the amount of food was increased as the animals grew. In the last experiment equal amounts of bread and milk were given to all the animals, but no attempt was made to control the exact amount of grain. In this experiment an amount of desiccated thymus equal to the amount of pineal powder was given to two of the control animals. The other control animals were given a corresponding amount of purified casein.

Observations were made in regard to all gross developmental changes such as appearance and texture of the coat, descent of testes and their appearance at various ages, state of nutrition, skeletal changes, body weight, and activities of the animals (the last two recorded weekly). The rats were killed at intervals of 3, 5, and 6 weeks after the pineal feeding had been begun. At autopsy measurements of body and tail length were made, and tissue was obtained from the endocrine glands and the organs of reproduction. The tissue was placed in formaldehyde-Zenker's solution, made with neutral formaldehyde. The sections were stained with hematoxylin and eosin for microscopic study.

## RESULTS.

During the entire series of experiments all the animals remained in an excellent state of nutrition. Although the powder of the pineal body was given in relatively large doses, it in no way affected their general health. The activities of the test animals equalled and cor-

[3] Donaldson, H. H., Memoirs of the Wistar Institute, No. 6, Philadelphia, 1915.

responded to those of the control animals. In no instance was any sexual precocity noted. The testes of the pineal-fed rats and their control rats descended almost simultaneously. Furthermore, no differences were noted in the appearance, size, or consistency of the testes of test and control animals of the same litters.

The results are summarized in Tables I to VIII and graphically presented in Text-figs. 1 to 5. There was essentially no variation between the weight of the pineal-fed animals and their respective controls. The pineal-fed males showed a difference in weight of 0.5 per

TABLE I.

*Gain in Weight of the Rats in Litter 1.*

Born Dec. 22, 1916. Six animals. Feeding begun Jan. 9, 1917. Age 18 days. Animals killed Feb. 13. Duration of experiment 35 days.

0.01 gm. of pineal powder given twice daily until Jan. 16; 0.02 gm. twice daily thereafter. Pineal powder given in very small amount of food to insure its ingestion. All animals given excess of wheat bread, corn bread, and milk; occasionally cracked corn and oats. Animals were all extremely healthy and vigorous throughout experiment. Testes descended about Jan. 27—age 36 days.

| Sex. | Animal. | Jan. 9. | Jan. 13. | Jan. 20. | Jan. 27. | Feb. 3. | Feb. 13. | Total gain. | Gain. |
|---|---|---|---|---|---|---|---|---|---|
| | | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *per cent* |
| M. | Control..... | 20.0 | 25.0 | 38.0 | 55.0 | 66.5 | 90.0 | 70.0 | 350 |
| F. | " ..... | 16.5 | 21.0 | 33.5 | 50.5 | 61.0 | 80.0 | 63.5 | 385 |
| M. | Pineal-fed..... | 17.5 | 22.0 | 32.0 | 46.0 | 58.0 | 75.5 | 58.0 | 331 |
| F. | " ..... | 17.0 | 22.0 | 34.5 | 46.0 | 55.0 | 67.0 | 50.0 | 294 |
| M. | " ..... | 18.5 | 22.5 | 34.5 | 50.0 | 56.5 | 75.5 | 57.0 | 308 |
| F. | " ..... | 17.0 | 22.0 | 35.5 | 49.5 | 57.0 | 74.0 | 57.0 | 335 |

cent less than their control animals and the females 9.6 per cent less than their control animals. Not only was there essentially no difference in the final weights of the two sets of rats but no striking variation was noted at any stage of the period of observation. In Litters 1 and 2 it will be seen that the percentage of weight gained by the control rats is somewhat greater than that gained by the pineal-fed animals. This is not true of Litters 3 and 4. Comparisons of the weights of the testes of the pineal-fed rats and their control animals and of the respective ratios of testicular weight to body weight show no difference beyond what may be considered the normal coefficient of varia-

TABLE II.

*Measurements and Weights of Organs of the Rats in Litter 1.*

| Sex. | Animal. | Body. | Tail. | Thymus. | Testes. | Ratio of testicular weight to body weight. |
|---|---|---|---|---|---|---|
| | | *cm.* | *cm.* | *gm.* | *gm.* | *per cent* |
| M. | Control | 15.5 | 12.9 | 0.312 | 1.447 | 1.60 |
| F. | " | 15.0 | 11.2 | 0.355 | | |
| M. | Pineal-fed | 14.9 | 11.9 | 0.205 | 1.412 | 1.87 |
| F. | " | 14.5 | 11.6 | 0.200 | | |
| M. | " | 14.8 | 12.2 | 0.235 | 1.050 | 1.39 |
| F. | " | 14.8 | 12.4 | 0.230 | | |

TABLE III.

*Gain in Weight of the Rats in Litter 2.*

Born Dec. 25, 1916. Six animals. Feeding begun Jan. 13, 1917. Age 18 days. Animals killed Feb. 3. Duration of experiment 22 days.

0.01 gm. of same powder that was used for Litter 1 given daily until Jan. 16; 0.02 gm. twice daily thereafter. Diet as in Table I. Animals were all healthy and vigorous throughout experiment. Testes descended about Jan. 27—age 33 days.

| Sex. | Animal. | Jan. 13. | Jan. 20. | Jan. 27. | Feb. 3. | Total gain. | Gain. |
|---|---|---|---|---|---|---|---|
| | | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *per cent* |
| M. | Pineal-fed | 12.0 | 23.0 | 40.5 | 47.0 | 35.0 | 292 |
| F. | " | 12.0 | 24.0 | 38.0 | 43.5 | 31.5 | 263 |
| " | " | 12.5 | 26.5 | 38.5 | 46.0 | 33.5 | 268 |
| " | " | 13.0 | 27.0 | 38.0 | 45.0 | 32.0 | 246 |
| M. | Control | 13.0 | 28.0 | 43.5 | 56.0 | 43.0 | 331 |
| F. | " | 13.5 | 27.5 | 43.5 | 55.5 | 42.0 | 311 |

TABLE IV.

*Measurements and Weights of Organs of the Rats in Litter 2.*

| Sex. | Animal. | Body. | Tail. | Thymus. | Testes. | Ratio of testicular weight to body weight. |
|---|---|---|---|---|---|---|
| | | *cm.* | *cm.* | *gm.* | *gm.* | *per cent* |
| M. | Pineal-fed | 12.2 | 10.2 | 0.156 | 0.469 | 0.998 |
| F. | " | 11.5 | 10.4 | 0.115 | | |
| " | " | 11.9 | 10.1 | 0.120 | | |
| " | " | 11.4 | 10.8 | 0.110 | | |
| M. | Control | 12.7 | 11.2 | 0.175 | 0.642 | 1.146 |
| F. | " | 12.4 | 11.4 | 0.175 | | |

TABLE V.

*Gain in Weight of the Rats in Litter 3.*

Born Feb. 1, 1917. Eight animals. Feeding begun Feb. 21. Age 21 days. Animals killed Apr. 5. Duration of experiment 43 days.

Fresh powdered desiccated pineal body from calves; first lot until Mar. 19, second lot until end of experiment. 0.0175 gm. of powder once daily until Feb. 24; 0.035 gm. once daily until Mar. 31; 0.035 gm. twice daily thereafter. Animals fed exactly equal amounts of milk and Graham flour. Food increased as the animals grew. Animals well throughout experiment, except for diarrhea which occurred intermittently during the experiment. This did not interfere with the vigor or playfulness of the animals. Testes descended about Mar. 18—age 46 days.

| Sex. | Animal. | Feb. 21. | Feb. 27. | Mar. 6. | Mar. 13. | Mar. 24. | Mar. 31. | Apr. 5. | Total gain. | Gain. |
|---|---|---|---|---|---|---|---|---|---|---|
| | | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *per cent* |
| M. | Control..... | 15.5 | 19.0 | 29.5 | 34.5 | 64.0 | 84.0 | 96.5 | 81.0 | 523 |
| F. | " ..... | 14.5 | 17.5 | 28.0 | 33.0 | 57.5 | 76.5 | 86.0 | 71.5 | 493 |
| M. | Pineal-fed.... | 15.0 | 17.0 | 27.0 | 33.0 | 62.0 | 85.5 | 98.5 | 83.5 | 557 |
| F. | " .... | 13.5 | 16.5 | 28.0 | 32.0 | 59.0 | 78.5 | 91.0 | 77.5 | 574 |
| M. | " .... | 14.5 | 17.5 | 29.0 | 33.5 | 62.5 | 85.5 | 102.5 | 88.0 | 607 |
| F. | " .... | 15.0 | 18.0 | 30.5 | 33.0 | 60.0 | 79.5 | 89.5 | 74.5 | 497 |
| M. | Control..... | 14.5 | 17.5 | 29.0 | 32.5 | 61.5 | 83.0 | 100.5 | 86.0 | 593 |
| F. | " ..... | 11.0 | 12.5 | 20.5 | 22.0 | 41.5 | 56.0 | 65.5 | 54.5 | 495 |

TABLE VI.

*Measurements and Weights of Organs of the Rats in Litter 3.*

| Sex. | Animal. | Body. | Tail. | Thymus. | Testes. | Ratio of testicular weight to body weight. |
|---|---|---|---|---|---|---|
| | | *cm.* | *cm.* | *gm.* | *gm.* | *per cent* |
| M. | Control..... | 16.3 | 13.2 | 0.260 | 1.840 | 1.907 |
| F. | " ..... | 14.7 | 13.9 | 0.241 | | |
| M. | Pineal-fed..... | 15.7 | 13.7 | 0.240 | 1.465 | 1.487 |
| F. | " ..... | 15.4 | 12.8 | 0.278 | | |
| M. | " ..... | 15.9 | 13.1 | 0.242 | 1.535 | 1.498 |
| F. | " ..... | 15.3 | 13.8 | 0.274 | | |
| M. | Control..... | 15.9 | 12.7 | 0.268 | 1.345 | 1.338 |
| F. | " ..... | 14.2 | 11.1 | 0.202 | | |

tion. The total testicular weight of the pineal-fed rats is 10 per cent less than that of the control rats. The measurements of body and tail length yield results similar to the results obtained in regard to

TABLE VII.

*Gain in Weight of the Rats in Litter 4.*

Born Mar. 10, 1917. Six animals. Feeding begun Mar. 31. Age 21 days. Animals killed May 5. Duration of experiment 35 days.

Same pineal powder used as in last part of experiment with Litter 3. 0.05 gm. of powder given twice daily. Same thymus as in contemporary thymus experiments. Animals given equal amounts of bread and milk. Health good except for bad infection with lice in middle of experiment, from which the male control suffered the most. All cured by sulfur ointment. Testes descended about Apr. 9—age 30 days.

| Sex. | Animal. | Mar. 31. | Apr. 7. | Apr. 14. | Apr. 21. | Apr. 28. | May 5. | Total gain. | Gain. |
|---|---|---|---|---|---|---|---|---|---|
| | | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *per cent* |
| M. | Control | 15.0 | 23.5 | 33.5 | 46.5 | 67.0 | 80.0 | 65.0 | 433 |
| F. | " | 18.0 | 24.5 | 38.0 | 53.0 | 78.0 | 93.0 | 75.0 | 417 |
| M. | Pineal-fed | 18.0 | 25.5 | 40.5 | 62.0 | 92.0 | 113.0 | 95.0 | 528 |
| F. | " | 17.0 | 28.5 | 44.5 | 61.5 | 88.5 | 105.0 | 88.0 | 518 |
| M. | Thymus-fed | 16.5 | 26.0 | 44.0 | 62.0 | 88.5 | 111.0 | 94.5 | 573 |
| F. | " | 19.0 | 25.5 | 42.5 | 59.0 | 84.0 | 96.0 | 77.0 | 405 |

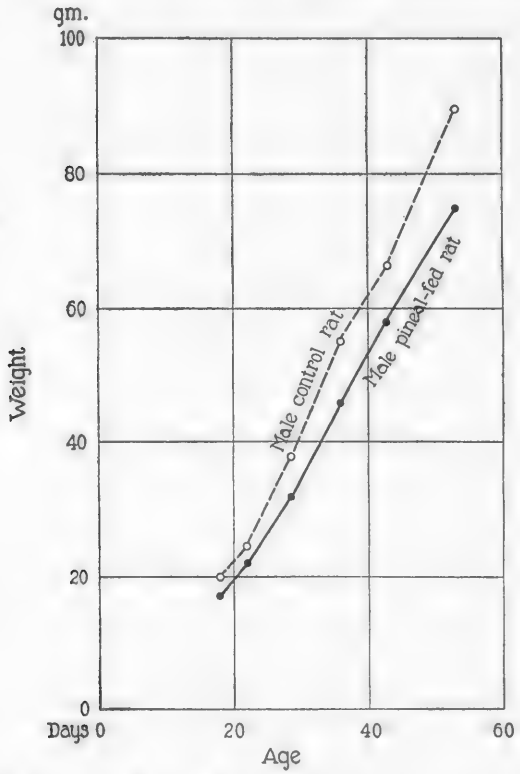
TABLE VIII.

*Measurements and Weights of Organs of the Rats in Litter 4.*

| Sex. | Animal. | Body. | Tail. | Thymus. | Testes. | Ratio of testicular weight to body weight. |
|---|---|---|---|---|---|---|
| | | *cm.* | *cm.* | *gm.* | *gm.* | *per cent* |
| M. | Control | 15.0 | 12.4 | 0.225 | 1.278 | 1.598 |
| F. | " | 15.5 | 14.3 | 0.240 | | |
| M. | Pineal-fed | 16.8 | 14.3 | 0.330 | 1.525 | 1.349 |
| F. | " | 16.2 | 13.5 | 0.355 | | |
| M. | Thymus-fed | 16.6 | 14.3 | 0.355 | 1.838 | 1.657 |
| F. | " | 15.9 | 14.2 | 0.255 | | |

weight; namely, that the body and tail lengths of the control rats of Litters 1 and 2 are greater than those of the pineal-fed animals and that no noteworthy differences between members of Litters 3 and 4

are present, provided the comparison is limited to members of the same litters. Furthermore, the results of the study of body weights and measurements are in accord with the observations frequently made in regard to the size of the animals. These observations showed that

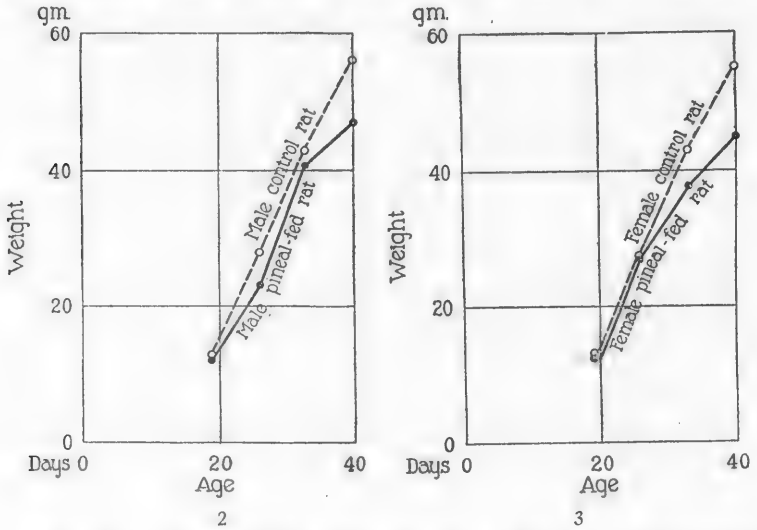



TEXT-FIG. 1. Curves showing the gain in weight of a male pineal-fed rat from Litter 1 and its control rat.

the difference in the size between the control and pineal-fed animals of the first two litters was slight, and no difference in members of the last two litters existed.

Microscopic studies of the endocrine glands of both control and pineal-fed animals revealed normal conditions; at least no differences

were discovered by the ordinary staining methods. Microscopic examination of the reproductive organs of the pineal-fed and control rats (male and female) likewise failed to reveal differences. All the results were entirely in accord with the normal findings in animals of the ages examined with the exception of the testes of the thymus-fed rats in Litter 4, which showed slightly more advanced spermatogenesis.



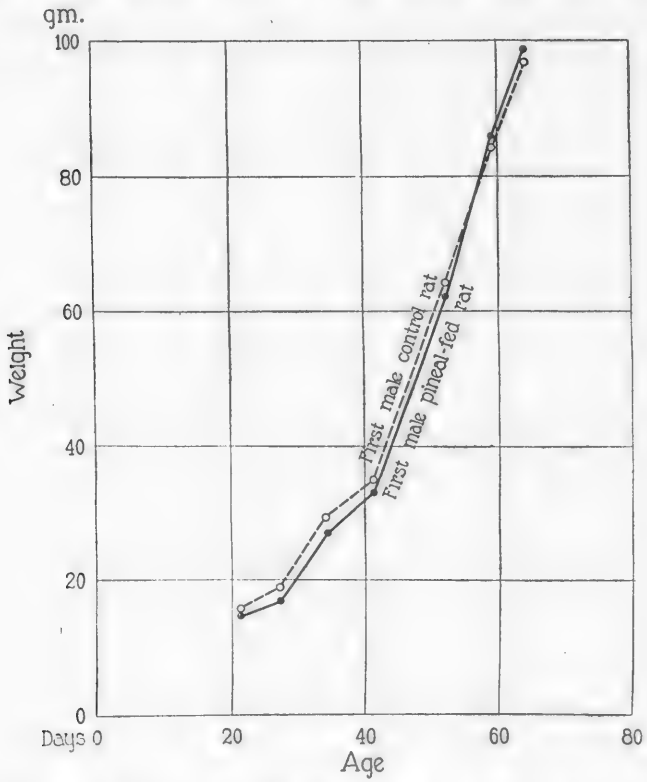

TEXT-FIGS. 2 and 3. Curves showing the gain in weight of a male pineal-fed and female pineal-fed rat from Litter 2 and their control rats.

## DISCUSSION.

Observation of the pineal-fed and control rats for differences in activity and gross developmental change failed to suggest the existence of any active principle in the pineal body. The statistics in regard to weight and body measurement and the microscopic findings, which represent the most accurate criteria of developmental changes, also are confirmatory of the view that the pineal body does not possess an internal secretion. It will be noted that in Litter 4 the pineal-fed animals showed a greater variation in weight than can be explained by the normal variation of the species. We ascribe this variation to

the severe skin infection from which these animals suffered, rather than to any specific effect of the pineal feeding.

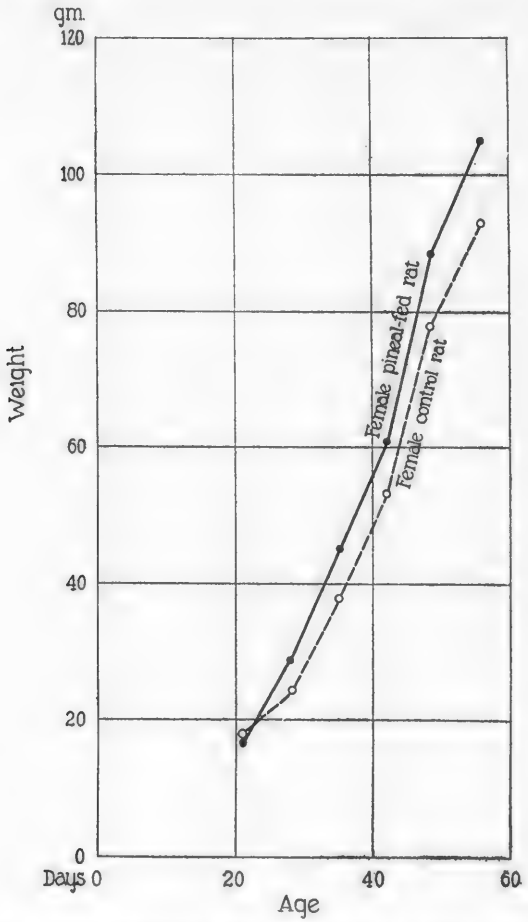
Special attention was given to any sexual changes that the pineal body might produce, because of the common belief that tumors of




TEXT-FIG. 4. Curves showing the gain in weight of a male pineal-fed rat from Litter 3 and its control rat.

this body cause sexual precocity and premature sexual development. Neither the activities of the test rats, nor the appearance, size, or weight of the testes, nor the time of their descent, nor the results of microscopic examination of either testes or ovaries suggested any relation between the pineal body and the reproductive organs.

There is little uniformity in the results obtained by previous workers who have studied the function of the pineal body. McCord[4] has expressed the opinion that




TEXT-FIG. 5. Curves showing the gain in weight of a female pineal-fed rat from Litter 4 and its control rat.

the pineal body "contains some substance capable of stimulating growth." He based his conclusion upon a review of the literature and experiments in which

[4] McCord, C. P., *Tr. Am. Gynec. Soc.*, 1917, xlii, 41.

either pineal extract was injected or the gland fed. Hoskins[5] concluded that the pineal body, when fed to albino rats, has no effect upon their development. Other observers, using a different method of study, also have obtained conflicting results. Dandy[6] removed the pineal body from young animals and was unable to note any developmental changes. Horrax,[7] on the other hand, using a similar method of extirpation, arrived at the conclusion that the pineal body stimulated growth and sexual development. Fenger[8] studied the physiological properties of the pineal body and found it essentially inactive. Park,[9] who made a study of the literature, states: "It seems probable that a mass of experimental literature of conflicting nature will develop about the pineal body, like that which has sprung up around the thymus and the spleen. It can now be said that the experimental work on the pineal up to the present time has failed to prove that it possesses a function."

An accurate interpretation of feeding experiments with gland substances demands that certain factors which may explain the conflicting results of various observers should be considered. In a previous study on the effect of hypophyseal feeding on growth, attention was called to the importance of using standardized stock such as the albino rat used in the present experiments.[10] As stated above, the normal development of this animal has been carefully studied and the statistics accumulated afford valuable data for comparison. The diet of the animal is another factor which may modify the results of feeding experiments. Little consideration has been given to the question of the adequacy of the diet in the past. We now know that not only adequate amounts of food elements are necessary but also that salts and so called food accessories have a direct relation to growth. It is important, therefore, that these substances should be given in equal amounts to control and test animals. Finally, mention should be made of the experimental method employed in its relation

[5] Hoskins, E. R., *J. Exp. Zool.*, 1916, xxi, 295.
[6] Dandy, W. E., *J. Exp. Med.*, 1915, xxii, 237.
[7] Horrax, G., *Arch. Int. Med.*, 1916, xvii, 627.
[8] Fenger, F., *J. Am. Med. Assn.*, 1916, lxvii, 1836.
[9] Park, E. A., *Am. J. Dis. Child.*, 1916, xii, 477.
[10] Work not yet published.

to the interpretation of the results. Oral administration of gland substances may be open to the criticism that the gland is broken down by digestive processes and thus becomes inactive. There is no evidence that such a change in the pineal body does or does not take place. The results of extirpation of the pineal body or injections of the gland may be modified by the trauma involved. The feeding method applied to the albino rat seemed to us the one most likely to reveal the true nature of pineal function.

## SUMMARY AND CONCLUSION.

The pineal body of young calves was fed to albino rats beginning at the age of 3 weeks and extending over periods of from 3 to 6 weeks. Four litters of rats were used. Of these, fourteen rats were fed the pineal powder and ten were used as control subjects. Observations in regard to developmental changes and microscopic examination of the endocrine glands and reproductive organs were made. The pineal-fed rats of the first two litters remained somewhat smaller than their controls. The pineal-fed and control animals of Litters 3 and 4 showed no differences in development. Microscopic studies showed no differences between pineal-fed and control rats.

We may therefore conclude that feeding the desiccated pineal body of young calves to young albino rats fails to produce any effect upon the early development of these animals.

## EXPLANATION OF PLATE 20.

FIG. 1. Photograph of (1) pineal-fed male, (2) male control, (3) pineal-fed female, and (4) female control rats of Litter 3. No appreciable difference in size can be noted between animals of the same sex.

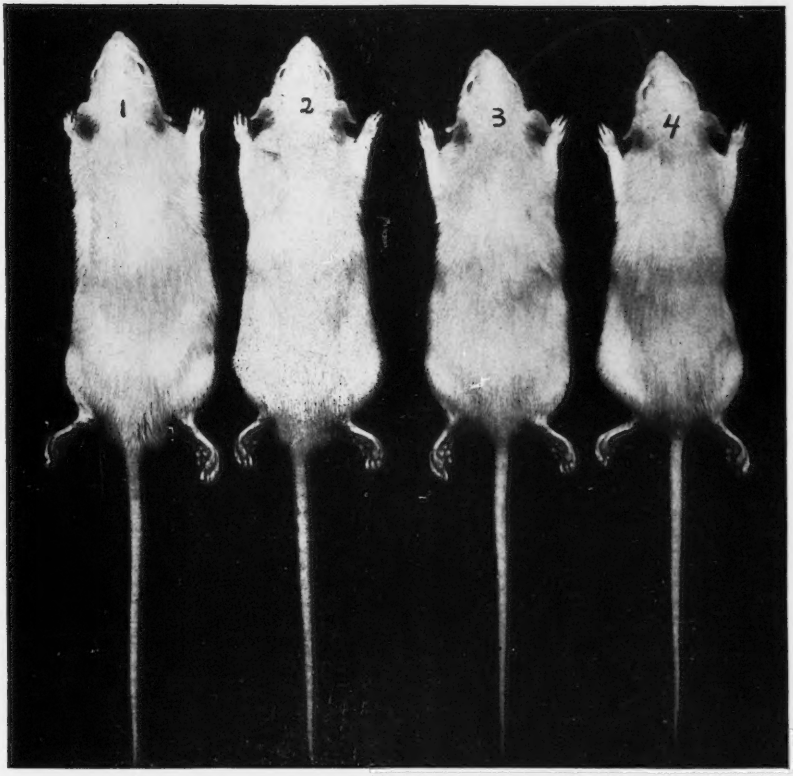



FIG. 1.

(Sisson and Finney: Effect of feeding pineal body on albino rat.)

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